CHROMATOGRAM

Retention time: 11 (Asp), 12 (Glu), 18 (Ser), 18.7 (Thr), 19.3 (Gly), 20 (Ala), 21.5 (Pro), 21.7 (Val), 22.2 (Arg), 23 (Met), 24 (Ile), 24.5 (Leu), 25 (Phe), 32.5 (ammonia), 34.5 (Lysine), 35 (His), 36.5 (Tyr)

KEY WORDS

derivatization

REFERENCE

Chang,J.-Y.; Knecht,R.; Braun,D.G. Amino acid analysis in the picomole range by precolumn derivatization and high-performance liquid chromatography, *Methods Enzymol.*, **1983**, *91*, 41–48.

SAMPLE

Matrix: peptides

Sample preparation: Heat 0.5-5 mg peptide with 1 mL 1% indolylpropionic acid in'6 M HCl under vacuum in a sealed tube at 115° for 4 h, evaporate to dryness, reconstitute with 10-100 mL water, remove a 1 mL aliquot, add 100 μL 20 mM dithioerythritol, add 100 μL 400 mM iodomethane in MeOH:water 50:50, add 200 μL 3 M NaOH, let stand for 10 min, add 200 μL 3 M HCl, mix briefly, add 400 μL 400 mM pH 10 sodium borate buffer, mix, check that pH is about 10. Remove a 100 μL aliquot, add 400 μL reagent, mix, let stand for 10 min, inject a 25 μL aliquot. (Iodomethane is used to protect cysteine as its S-methyl derivative. Prepare reagent by dissolving 30 mg o-phthaldialdehyde in 1 mL EtOH, add 22 mL 400 mM pH 10 sodium borate buffer, add 30 mg Boc-L-cysteine. Prepare Boc-L-cysteine by adding Boc-S-benzyl-L-cysteine to liquid ammonia, add metallic sodium until a blue color persists for 15 min, add ammonium chloride until all the excess sodium is destroyed, allow the ammonia to evaporate, add ice and water, extract with ethyl acetate.)

HPLC VARIABLES

Column: 120 × 4 3 μm Hypersil ODS

Mobile phase: Gradient. A was 50 mM pH 7.0 phosphate buffer. B was MeOH:THF:50 mM pH 7.0 phosphate buffer 65:1:35. A:B from 70:30 to 0:100 over 48 min.

Flow rate: 1.4

Injection volume: 25

Detector: F ex 344 em 443

CHROMATOGRAM

Retention time: 20.5 (L-Thr), 22.5 (D-Thr), 28.5 (S-methyl-L-Cys), 31 (L-threoninol), 31.5 (S-methyl-D-Cys), 32 (D-threoninol), 34 (L-Trp), 36.5 (L-Phe), 37 (D-Trp), 38 (D-Phe), 47 (L-Lys), 48 (D-Lys)

KEY WORDS

derivatization; chiral; comparison with the results obtained with other thiols in the derivatization reagent

REFERENCE

Buck,R.H.; Krummen,K. High-performance liquid chromatographic determination of enantiomeric amino acids and amino alcohols after derivatization with *o*-phthaldialdehyde and various chiral mercaptans. Application to peptide hydrolysates, *J.Chromatogr.*, **1987**, *387*, 255–265.

SAMPLE

Matrix: peptides

Sample preparation: Mix ≤5 mg peptide and 200 μL 6 M HCl, heat at 100° for 6 h, evaporate to dryness under a stream of nitrogen at 80-100°, reconstitute with 3 M HCl in isopropanol, heat at 105° for 20 min, evaporate to dryness under a stream of nitrogen at 105°, reconstitute with 1 mL 5 mg/mL sodium heptanesulfonate in water, extract with 5 mL dichloromethane. Evaporate the organic layer to dryness under a stream of nitrogen

at 50°, add 20 mg anhydrous sodium carbonate, add a 3-fold molar excess of 1 mg/mL S-flunoxaprofen chloride in dichloromethane or ethyl acetate, heat at 40° for 1 h (or agitate moderately overnight), evaporate to dryness, reconstitute with 200 μL mobile phase, inject a 10-20 μL aliquot. (Prepare S-flunoxaprofen chloride as follows. Dissolve 1 mmole S-flunoxaprofen in 25 mL toluene, add a trace of DMF (this paper), add 2.5 mL thionyl chloride, reflux for 30 min, remove solvent by evaporation, dry the residue under vacuum over KOH, recrystallize from dichloromethane (mp 73°) (J.Chromatogr. 1988, 427, 131).)

HPLC VARIABLES

Column: 250 × 4.6 5 μm Zorbax-Sil
Mobile phase: n-Hexane:chloroform:EtOH 100:10:1
Flow rate: 2
Injection volume: 10-20
Detector: UV 305 or F ex 305 em 355

CHROMATOGRAM

Retention time: k' 11.2 (D-alanine), k' 15.2 (L-alanine), k' 19.5 (glycine), k' 4.8 (D-isoleucine), k' 4.5 (L-isoleucine), k' 7.1 (D-leucine), k' 6.0 (L-leucine), k' 11.9 (D-methionine), k' 10.5 (L-methionine), k' 6.6 (D-phenylalanine), k' 6.4 (L-phenylalanine), k' 15.0 (D-proline), k' 12.3 (L-proline), k' 22.6 (D-tyrosine), k' 13.1 (L-tyrosine)
Limit of detection: 0.1-0.5 ng

KEY WORDS

normal phase; derivatization; chiral

REFERENCE

Langguth,P.; Spahn,H.; Merkle,H.P. Fluorescence assay for small peptides and amino acids: high-performance liquid chromatographic determination of selected substrates using activated *S*-flunoxaprofen as a chiral derivatizing agent, *J.Chromatogr.*, **1990**, *528*, 55–64.

SAMPLE

Matrix: peptides
Sample preparation: Dry 0.5-1 nmole peptide in a tube. add 500 μL 6 M HCl, evacuate, flush with nitrogen, evacuate, seal, heat in the vapor phase at 110° for 20-24 h, evaporate to dryness under reduced pressure, reconstitute with 5 μL 200 mM sodium bicarbonate, dry under reduced pressure, add 5 μL water, add a 2- to 3-fold molar excess of reagent in 10 μL acetone, heat in the dark with gentle shaking at 50° for 1 h, evaporate to dryness under reduced pressure, reconstitute, inject an aliquot. (Synthesize the reagent, 2,4-dinitrophenyl-1-fluoro-5-L-alanine, as follows. Add 10 mL 200 mM 1,5-difluoro-2,4-dinitrobenzene in acetone dropwise with stirring to 10 mM 200 mM L-alanine in 5 mM sodium bicarbonate, stir in the dark at room temperature at pH 8 for 4 h, acidify to pH 3 with 2 M HCl, evaporate to dryness, take up in 100 mM sodium bicarbonate, extract with diethyl ether, crystallize by the addition of hot 2 M HCl, filter, wash with acidified water, dry in the air in the dark to give 2,4-dinitrophenyl-1-fluoro-5-L-alanine as golden-yellow scales (mp 136-137°).)

HPLC VARIABLES

Column: 250 × 2 5 μm Ultrasphere octyl
Mobile phase: Gradient. A was 40 mM pH 2.2 triethylammonium phosphate. B was MeCN: isopropanol 80:20. A:B from 76:24 to 61:39 over 38 min, to 35:65 over 32 min, to 5:95 over 0.2 min, maintain at 5:95 for 3.8 min, return to initial conditions over 0.2 min.
Column temperature: 25
Flow rate: 0.2
Injection volume: 20
Detector: UV 340

CHROMATOGRAM

Retention time: 7 (D-His), 8 (L-His), 9.5 (D-Arg), 10 (L-Arg), 17 (L-Ser), 18 (D-Ser), 19 (L-Thr), 20.5 (L-Asp), 21.5 (D-Asp), 23.5 (L-Glu), 24.5 (D-Glu), 25 (D-Thr), 25.5 (Gly), 29.5 (ammonia), 30 (L-Ala), 37 (D-Ala), 40.5 (L-Met), 43.5 (L-Val), 44.5 (L-norvaline), 49 (D-Met), 50.5 (L-Phe), 51 (L-Ile), 51.5 (L-Leu), 52 (L-Lys, D-Val), 55 (D-Lys), 56 (D-Phe), 57.5 (D-Ile), 58.5 (D-Leu), 60 (L-Tyr), 65 (D-Tyr)

KEY WORDS

derivatization; chiral

REFERENCE

Scaloni,A.; Simmaco,M.; Bossa,F. Determination of the chirality of amino acid residues in the course of subtractive Edman degradation of peptides, *Anal.Biochem.*, **1991**, *197*, 305–310.

SAMPLE

Matrix: peptides

Sample preparation: Heat 60 nmole peptide with 100 μL acetic anhydride at 50° for 10 min, evaporate to dryness under reduced pressure, reconstitute with 100 μL water:triethylamine 98:2, let stand at 25° for 5 min, evaporate to dryness under reduced pressure, reconstitute with 40 μL anhydrous MeCN, add 10 μL pyridine, add 60 μL 1 mM diphenyl phosphoroisothiocyanate in MeCN, heat at 50° for 30 min, evaporate to dryness under reduced pressure, reconstitute with 100 μL 0.1% trifluoroacetic acid in water, inject an aliquot. (Synthesis of diphenyl phosphoroisothiocyanate is as follows. Add 50.1 g diphenyl chlorophosphate to a solution of 19.3 g potassium thiocyanate in 200 mL MeCN, shake for 3 h, let stand for 3 h, dilute with 300 mL dry benzene (Caution! Benzene is a carcinogen!), filter, evaporate the filtrate, distil the residue to give diphenyl phosphoroisothiocyanate (bp 105°/0.1 mm Hg) (J. Chem. Soc. 1953, 673).)

HPLC VARIABLES

Column: 250 × 2.0 3 μm Reliasil C18 (Column Engineering, Ontario CA)

Mobile phase: Gradient. A was 0.1% trifluoroacetic acid in water. B was MeCN:MeOH: water 80:10:10. A:B 100:0 for 2 min, to 96:4 over 3 min, to 65:35 over 35 min, to 50:50 over 10 min, return to initial conditions over 2 min.

Detector: UV 265

CHROMATOGRAM

Retention time: 7 (asparagine), 8 (glycine), 10 (aspartic acid), 12.5 (glutamine), 13.5 (histidine), 14 (alanine), 15.5 (glutamic acid), 17 (serine), 19 (arginine), 21 (cysteine), 23 (lysine), 23.5 (threonine), 26 (valine), 28 (tyrosine), 28.5 (methionine), 35.5 (isoleucine), 38 (leucine), 41 (phenylalanine), 44 (tryptophan)

KEY WORDS

derivatization

REFERENCE

Bailey,J.M.; Nikfarjam,F.; Shenoy,N.R.; Shively,J.E. Automated carboxy-terminal sequence analysis of peptides and proteins using diphenyl phosphoroisothiocyanatidate, *Protein Sci.*, **1992**, *1*, 1622–1633.

SAMPLE

Matrix: peptides

Sample preparation: Dry a solution containing 1 μg peptide under vacuum, add 200 μL 0.06% phenol in constant-boiling HCl, evacuate and purge with nitrogen 3 times, evacuate and seal, heat at 114° for 22 h, cool, evaporate to dryness under reduced pressure, reconstitute with 20 μL 20 mM HCl, add 60 μL 200 mM pH 8.8 sodium borate containing 5 mM EDTA, add 20 μL 3 mg/mL 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) in MeCN, inject an aliquot. (6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate can be purchased from Waters or synthesized as follows. Reflux 3 g N,N'-succinimidyl carbonate in

100 mL dry MeCN, add 1.5 g 6-aminoquinoline in 50 mL dry MeCN dropwise over 30 min, reflux for 30 min, evaporate to half volume under reduced pressure, cool for 24 h, filter, wash the solid with cold MeCN, recrystallize from MeCN to obtain 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as off-white crystals (mp 210-215° (d)) (Anal. Biochem. 1993, 211, 279).)

HPLC VARIABLES

Column: 150 × 3.9 Nova-Pak C18

Mobile phase: Gradient. MeCN:buffer:water 0:100:0 for 0.5 min, to 1:99:0 (step gradient), to 5:95:0 over 17.5 min, to 9:91:0 over 1 min, to 17:83:0 over 10.5 min, to 32:68:0 over 3.5 min, to 60:0:40 (step gradient), maintain at 60:0:40 for 3 min, re-equilibrate at initial conditions for 9 min. (Buffer was 140 mM sodium acetate containing 17 mM triethylamine and 3 mM EDTA, adjusted to pH 5.05 with phosphoric acid.)

Column temperature: 37

Flow rate: 1

Detector: F ex 250 em 395

CHROMATOGRAM

Retention time: 14 (Asp), 15 (Ser), 16 (Glu), 17 (Gly), 17.5 (His), 19 (ammonia), 21.8 (Arg), 22 (Thr), 23 (Ala), 24 (Pro), 26.8 (Cys), 27 (Tyr), 28.3 (Val), 28.7 (Met), 30.7 (Lys), 31.8 (Ile), 32.5 (Leu), 33.5 (Phe)

Limit of detection: 38-794 fmole

KEY WORDS

derivatization; procedure can also be used to derivatize peptides; changes to the gradient are required.

REFERENCE

De Antonis,K.M.; Brown,P.R.; Cohen,S.A. High-performance liquid chromatographic analysis of synthetic peptides using derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, *Anal.Biochem.*, **1994**, *223*, 191–197.

SAMPLE

Matrix: peptides

Sample preparation: Lyophilize 100 μL peptide solution, add 100 μL 6 M HCl, heat at 105° in a sealed glass tube for 16 h, lyophilize 50 μL of the hydrolyzate, reconstitute with 15 μL 200 mM pH 8.5 sodium bicarbonate solution, add 15 μL 0.015% acenaphthene-5-sulfonyl chloride in dry acetone, mix, heat at 47° for 30 min, cool, add 100 μL 2% ethylamine, inject a 20 μL aliquot. (Preparation of acenaphthene-5-sulfonyl chloride is as follows. Dissolve 20 g acenaphthene in 100 g nitrobenzene, cool to 0°, add 9 mL chlorosulfonic acid dropwise with stirring, maintain the temperature below 5°, when the addition is complete allow the temperature to rise to 20° over 30 min, add 500 mL water. Remove the aqueous layer and neutralize it with solid sodium carbonate, heat and add NaCl until precipitation occurs, cool in an ice bath for 1 h, filter, heat at 140° to remove traces of water and nitrobenzene to give acenaphthene-5-sulfonic acid sodium salt as a pale yellow solid (mp >300°). Grind 10 g acenaphthene-5-sulfonic acid sodium salt with 3.5 g phosphorus pentachloride in a mortar for 3 min, add ice and water, extract with 100 mL ethyl acetate. Wash the ethyl acetate layer with 5% sodium bicarbonate and with water until neutral, dry over anhydrous sodium sulfate, evaporate the ethyl acetate under a stream of nitrogen, chromatograph on a 300 × 20 column of silica gel H with toluene to give acenaphthene-5-sulfonyl chloride (mp 98-101°) as the first yellow band to elute.)

HPLC VARIABLES

Column: 250 × 4.6 5 μm Hypersil C18

Mobile phase: Gradient. A was THF:10 mM pH 4.2 acetate buffer 5:95. B was THF:MeCN 10:90. A:B from 90:10 to 60:40 over 30 min, maintain at 60:40 for 15 min, 0:100 for 3 min.

Flow rate: 1

Injection volume: 20
Detector: F ex 230 em 420

CHROMATOGRAM

Retention time: 7.5 (asparagine), 8.5 (glycine), 9.5 (serine), 10 (glutamine), 11 (alanine), 12.5 (arginine), 18.5 (ethylamine), 22.5 (valine), 26.5 (proline), 28 (tryptophan), 32.5 (lysine), 40 (tyrosine)

KEY WORDS

derivatization

REFERENCE

Gifford,L.A.; Owusu-Daaku,F.T.K.; Stevens,A.J. Acenaphthene fluorescence derivatization reagents for use in high-performance liquid chromatography, *J.Chromatogr.A*, **1995**, *715*, 201–212.

SAMPLE

Matrix: peptides
Sample preparation: Mix 10 μL of a solution containing 50 pmole peptide with 35 μL reagent, heat at 50° for 10 min, evaporate to dryness at 50° in a centrifugal evaporator for 5 min, reconstitute with 10 μL water, wash with three 100 μL portions of n-heptane: dichloromethane 90:10, evaporate to dryness at 50° in a centrifugal evaporator for 15 min, reconstitute with 30 μL trifluoroacetic acid, heat at 50° for 5 min, evaporate to dryness under a stream of nitrogen. Reconstitute with 20 μL water, add 100 μL n-heptane:dichloromethane 70:30, mix, centrifuge at 1000 g for 5 min, repeat extraction 3 times. (The aqueous phase contains the residual peptide and can be subjected to the same procedure to determine the next amino acid.) Combine the organic layers and evaporate them to dryness under a stream of nitrogen, reconstitute with 20 μL water:trifluoroacetic acid 80: 20, heat at 50° for 10 min, dry under a stream of nitrogen, reconstitute with mobile phase, inject an aliquot. (Reagent was phenylisothiocyanate:EtOH:pyridine 1:4:2.)

HPLC VARIABLES

Column: 150 × 6 5 μm ES-1/2phCD (β-cyclodextrin 50% modified with phenylcarbamoyl) (Shinwakakou, Kyoto) (Column A) or 150 × 6 5 μm Ultron ES-phCD (phenylcarbamoylated β-cyclodextrin) (Shinwakakou, Kyoto) (Column B)
Mobile phase: MeOH:water 25:75 containing 10 mM formic acid (Mobile Phase A) or MeCN:MeOH:water 10:45:45 containing 10 mM formic acid (Mobile Phase B)
Flow rate: 0.7
Detector: UV 269

CHROMATOGRAM

Retention time: 15 (Gly) (Column A, Mobile Phase A), 16 (L-Ala) (Column A, Mobile Phase A), 17 (D-Ala) (Column A, Mobile Phase A), 17 (D-Leu) (Column B, Mobile Phase B), 17.5 (L-Leu) (Column B, Mobile Phase B), 21 (D-Phe) (Column B, Mobile Phase B), 22.5 (L-Tyr) (Column A, Mobile Phase A), 23 (L-Phe) (Column B, Mobile Phase B), 24 (D-Tyr) (Column A, Mobile Phase A)

KEY WORDS

chiral; derivatization

REFERENCE

Imai,K.; Matsunaga,H.; Santa,T.; Homma,H. Availability of phenylisothiocyanate for the amino acid sequence/configuration determination of peptides containing D/L-amino acids, *Biomed.Chromatogr.*, **1995**, *9*, 195–196.

SAMPLE

Matrix: peptides
Sample preparation: Vortex 10 μL of a 50-100 μM solution of a dipeptide in pyridine: water 50:50 with 10 μL 20 mM 7-N,N-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl)

isothiocyanate in pyridine:water 50:50, heat at 50° for 15 min, wash 3 times with 100 μL portions of n-heptane:dichloromethane 80:20. Evaporate the aqueous layer to dryness at 50° for 15 min, add 30 μL 1% boron trifluoride in dichloromethane containing 0.02% ethanethiol, heat at 50° for 5 min, evaporate to dryness under a stream of nitrogen, add 20 μL water, add 100 μL n-heptane:dichloromethane 70:30, mix, centrifuge at 1000 g for 5 min, repeat the extraction 3 times. Combine the organic layers and evaporate them to dryness under a stream of nitrogen, reconstitute the residue in MeCN, inject an aliquot. (Synthesis of 7-N,N-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate is as follows. Dissolve 0.5 g magnesium sulfate heptahydrate and 6 g NaOH in 60 mL water, throughout the reaction keep the flask at about 20° with cold water cooling, add 15 mL 30% hydrogen peroxide, add 75 mL MeOH, add 12.1 g powdered benzoyl peroxide in one go, stir for 10 min, pour into 150 mL 20% sulfuric acid, extract three times with 50 mL portions of chloroform, determine peroxybenzoic acid concentration by iodometric titration (Tetrahedron 1967, 23, 3327). Slowly add 110 mL 1 M peroxybenzoic acid in chloroform to 7 g 2,6-difluoroaniline dissolved in 100 mL chloroform, stir at room temperature, when reaction is complete (iodometric titration) wash with 2% sodium thiosulfate, wash with 5% sodium carbonate, wash with water, dry over anhydrous sodium sulfate, evaporate to dryness under reduced pressure, recrystallize 2,6-difluoronitrosobenzene form EtOH (mp 108.5-109.5). Stir 8.5 g 2,6-difluoronitrosobenzene in 85 mL DMSO at room temperature and add a solution of 3.91 g sodium azide in 85 mL DMSO dropwise, let stand for about 1 h, add to a large volume of water, extract with ether, dry the extracts over anhydrous sodium sulfate, evaporate to dryness under reduced pressure and distil to give 4-fluoro-2,1,3-benzoxadiazole as a colorless oil (bp 83°/12 mm Hg) (J.Chem.Soc.(C) 1970, 1433). Add 11 mL chlorosulfonic acid dropwise to 3 g 4-fluoro-2,1,3-benzoxadiazole in 10 mL chloroform at 0-10° (use a calcium chloride drying tube), stir at room temperature for 1 h, reflux for 2 h, cool, slowly pour into ice water, remove the organic layer, extract the aqueous layer with chloroform, combine the organic layer, wash, dry over anhydrous magnesium sulfate, evaporate under reduced pressure, take up the residue in 5 mL benzene (Caution! Benzene is a carcinogen!), chromatograph on a 150 × 30 column of silica gel (100-200 mesh Kanto Chemical) with n-hexane:benzene 50:50, evaporate the appropriate fractions to give 4-(chlorosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (CBD-F) as pale yellow needles (mp 64-66°) (Anal. Chem. 1984. 56, 2461). Stir 0.76 g CBD-F in 70 mL MeCN at 0-10° and add 1 g dimethylamine hydrochloride in 10 mL 100 mM pH 10 borax dropwise, adjust pH to 5 with 1 M HCl, concentrate to about 10 mL under reduced pressure, extract three times with 200 mL portions of diethyl ether, wash with water, dry over anhydrous magnesium sulfate, evaporate under reduced pressure, chromatograph on a 500 × 20 column of silica gel with chloroform, isolate the appropriate fraction and re-chromatograph on the same column with ethyl acetate:benzene 1:2 to give 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) as white needles (mp 124-125°) (yield = 1% !). On a Merck no. 5714 $60F_{254}$ tlc plate eluted with chloroform DBD-F has Rf 0.32 and lies between two other reaction products (Analyst 1989, 114, 413). It is also reported that DBD-F can be purchased from Tokyo Kasei. Add 100 μL 28% ammonia in water to 50 mg 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole in 15 mL MeCN, stir at room temperature overnight, filter, evaporate the filtrate to dryness under reduced pressure, recrystallize from MeCN to give 4-amino-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole as pale yellow needles (mp 214-217°). Add 1 mL 30% thiophosgene in benzene dropwise to 200 mg 4-amino-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole in 15 mL MeCN, reflux for 5 h, concentrate under reduced pressure, extract the residue twice with 20 mL portions of chloroform. Combine the chloroform layers, filter, evaporate the filtrate to dryness, chromatograph the residue on 15 g silica gel G-200 with chloroform. Collect the fraction containing the product and evaporate it to dryness under reduced pressure, recrystallize from benzene/n-hexane to obtain 7-N,N-dimethylaminosulfonyl-4-(2,1,3- benzoxadiazolyl) isothiocyanate as pale yellow-white crystals (mp 122-124°; yield 26%) (Biomed. Chromatogr. 1993, 7, 56).)

HPLC VARIABLES
Column: 150 × 6 5 μm ES-1/4phCD phenylcarbamoylated β-cyclodextrin (Shinwa, Kyoto)
Mobile phase: MeCN:MeOH:water 15:40:45 containing 10 mM acetic acid
Injection volume: 20

Detector: F ex 387 em 524

CHROMATOGRAM

Retention time: 21 (D-Pro), 23 (L-Pro), 27 (L-Leu), 30 (D-Leu)

KEY WORDS

derivatization; chiral

REFERENCE

Matsunaga,H.; Iida,T.; Fukushima,T.; Santa,.; Homma,H.; Imai,K. Boron-trifluoride etherate (Lewis acid) as an efficient acid at cyclization/cleavage reaction of D/L-amino acids affording the retention of their original configuration in the Edman sequencing method of peptides, *Biomed.Chromatogr.*, **1996**, *10*, 95–96.

SAMPLE

Matrix: peptides

Sample preparation: Mix 10 μL of a 50 μM peptide solution in pyridine:water 50:50 with 10 μL 20 mM 7-N,N-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate in pyridine:water 50:50, vortex, heat at 50° for 15 min, wash three times with 100 μL portions of n-heptane:dichloromethane 80:20, dry at 50° under reduced pressure for 15 min, add 30 μL 1% boron trifluoride etherate in dichloroethane containing 0.1% ethanethiol, heat at 50° for 5 min, evaporate to dryness under a stream of nitrogen, add 20 μL water, add 100 μL ethyl acetate:benzene 75:25 (Caution! Benzene is a carcinogen!), mix, centrifuge at 1000 g for 1 min, repeat the extraction 3 times. Combine the organic layers and evaporate them to dryness under a stream of nitrogen, reconstitute the residue in mobile phase, inject an aliquot. (Evaporate the aqueous phase to dryness and continue the cycle. Boron trifluoride gives less racemization than trifluoroacetic acid. Synthesis of 7-N,N-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate is as follows. Dissolve 0.5 g magnesium sulfate heptahydrate and 6 g NaOH in 60 mL water, throughout the reaction keep the flask at about 20° with cold water cooling, add 15 mL 30% hydrogen peroxide, add 75 mL MeOH, add 12.1 g powdered benzoyl peroxide in one go, stir for 10 min, pour into 150 mL 20% sulfuric acid, extract three times with 50 mL portions of chloroform, determine peroxybenzoic acid concentration by iodometric titration (Tetrahedron 1967, 23, 3327). Slowly add 110 mL 1 M peroxybenzoic acid in chloroform to 7 g 2,6-difluoroaniline dissolved in 100 mL chloroform, stir at room temperature, when reaction is complete (iodometric titration) wash with 2% sodium thiosulfate, wash with 5% sodium carbonate, wash with water, dry over anhydrous sodium sulfate, evaporate to dryness under reduced pressure, recrystallize 2,6-difluoronitrosobenzene form EtOH (mp 108.5-109.5). Stir 8.5 g 2,6-difluoronitrosobenzene in 85 mL DMSO at room temperature and add a solution of 3.91 g sodium azide in 85 mL DMSO dropwise, let stand for about 1 h, add to a large volume of water, extract with ether, dry the extracts over anhydrous sodium sulfate, evaporate to dryness under reduced pressure and distil to give 4-fluoro-2,1,3-benzoxadiazole as a colorless oil (bp 83°/12 mm Hg) (J.Chem.Soc.(C) 1970, 1433). Add 11 mL chlorosulfonic acid dropwise to 3 g 4-fluoro-2,1,3-benzoxadiazole in 10 mL chloroform at 0-10° (use a calcium chloride drying tube), stir at room temperature for 1 h, reflux for 2 h, cool, slowly pour into ice water, remove the organic layer, extract the aqueous layer with chloroform, combine the organic layer, wash, dry over anhydrous magnesium sulfate, evaporate under reduced pressure, take up the residue in 5 mL benzene (Caution! Benzene is a carcinogen!), chromatograph on a 150 × 30 column of silica gel (100-200 mesh Kanto Chemical) with n-hexane:benzene 50:50, evaporate the appropriate fractions to give 4-(chlorosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (CBD-F) as pale yellow needles (mp 64-66°) (Anal. Chem. 1984. 56, 2461). Stir 0.76 g CBD-F in 70 mL MeCN at 0-10° and add 1 g dimethylamine hydrochloride in 10 mL 100 mM pH 10 borax dropwise, adjust pH to 5 with 1 M HCl, concentrate to about 10 mL under reduced pressure, extract three times with 200 mL portions of diethyl ether, wash with water, dry over anhydrous magnesium sulfate, evaporate under reduced pressure, chromatograph on a 500 × 20 column of silica gel with chloroform, isolate the appropriate fraction and re-chromatograph on the same column with ethyl acetate:benzene 1:2 to give 4-(N,N-dimethylami-

nosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) as white needles (mp 124-125°) (yield = 1% !). On a Merck no. 5714 $60F_{254}$ tlc plate eluted with chloroform DBD-F has Rf 0.32 and lies between two other reaction products (Analyst 1989, 114, 413). It is also reported that DBD-F can be purchased from Tokyo Kasei. Add 100 μL 28% ammonia in water to 50 mg 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole in 15 mL MeCN, stir at room temperature overnight, filter, evaporate the filtrate to dryness under reduced pressure, recrystallize from MeCN to give 4-amino-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole as pale yellow needles (mp 214-217°). Add 1 mL 30% thiophosgene in benzene dropwise to 200 mg 4-amino-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole in 15 mL MeCN, reflux for 5 h, concentrate under reduced pressure, extract the residue twice with 20 mL portions of chloroform. Combine the chloroform layers, filter, evaporate the filtrate to dryness, chromatograph the residue on 15 g silica gel G-200 with chloroform. Collect the fraction containing the product and evaporate it to dryness under reduced pressure, recrystallize from benzene/n-hexane to obtain 7-N,N-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate as pale yellow-white crystals (mp 122-124°; yield 26%) (Biomed. Chromatogr. 1993, 7, 56).)

HPLC VARIABLES

Column: 150 × 6 5 μm ES-1/4phCD 25% phenylcarbamoylated β-cyclodextrin (Shinwa)
Mobile phase: MeOH:water 70:30 containing 10 mM acetic acid
Column temperature: 5
Flow rate: 0.5
Injection volume: 20
Detector: F ex 387 em 524

CHROMATOGRAM

Retention time: 19 (L-Arg), 21 (D-Arg), 30 (L-Phe), 33 (D-Phe), 35 (L-Met), 38 (D-Met)

KEY WORDS

derivatization; chiral

REFERENCE

Matsunaga,H.; Santa,T.; Iida,T.; Fukushima,T.; Homma,H.; Imai,K. Proton: A major factor for the racemization and the dehydration at the cyclization/cleavage stage in the Edman sequencing method, *Anal.Chem.*, **1996**, *68*, 2850–2856.

SAMPLE

Matrix: perfusate
Sample preparation: Dry 120 μL perfusate under vacuum, add 50 μL EtOH:water:triethylamine 40:40:20, vortex, dry under vacuum, add 20 μL EtOH:water:triethylamine: phenylisothiocyanate 70:10:10:10, vortex, let stand at room temperature for 20 min, vacuum dry for at least 8 h (most of the derivatizing reagent must be removed in the first hour), reconstitute with 100 μL mobile phase A, inject a 70 μL aliquot.

HPLC VARIABLES

Guard column: 30-40 μm pellicular RP18
Column: 150 × 3.9 Novapak C18
Mobile phase: Gradient. A was MeCN:14 mM sodium acetate:triethylamine 4.5:95.5:0.05, adjusted to pH 6.6 with glacial acetic acid. B was MeCN:water 60:40. A:B 100:0 for 1 min, to 95:5 (step gradient), maintain at 95:5 for 9 min, to 94:6 (step gradient), to 0:100 over 4 min, maintain at 0:100 for 4 min, return to initial conditions, re-equilibrate for 10 min.
Column temperature: 37
Flow rate: 1.1 for 10 min, to 1.5 over 4 min, maintain at 1.5
Injection volume: 70
Detector: UV 254

CHROMATOGRAM
Retention time: 2 (aspartate), 2.4 (glutamate), 3.1 (adenosine), 3.4 (hydroxyproline), 5 (glutamine), 6.4 (taurine), 6.7 (gamma-aminobutyric acid), 9 (kainic acid)

KEY WORDS
derivatization

REFERENCE
Rogers,K.L.; Philibert,R.A.; Allen,A.J.; Molitor,J.; Wilson,E.J.; Dutton,G.R. HPLC analysis of putative amino acid neurotransmitters released from primary cerebellar cultures, *J.Neurosci.Methods*, **1987**, *22*, 173–179.

SAMPLE
Matrix: perfusate
Sample preparation: Lyophilize perfusate, reconstitute with water. Remove a 400 μL aliquot and add it to 100 μL 100 mM pH 6.2 borate buffer, adjust pH to 7.7 with 100 mM NaOH, add 500 μL 15.4 mM N-(9-fluorenylmethoxycarbonyloxy)succinimide (9-fluorenylmethyl N-succinimidyl carbonate) in acetone, let stand at room temperature for 1 min, extract twice with 2 mL portions of pentane. Evaporate the aqueous layer to dryness under reduced pressure, reconstitute with 200 μL water, filter (0.45 μm), inject a 10 μL aliquot of the filtrate.

HPLC VARIABLES
Column: 250 × 4.6 5 μm Spherisorb ODS II
Mobile phase: Gradient. MeCN:35 mM pH 5.5 ammonium acetate from 20:80 to 25:75 over 10 min, maintain at 25:75 for 5 min, to 50:50 over 25 min, to 80:20 over 10 min.
Flow rate: 1
Injection volume: 10
Detector: F ex 254 em 313

CHROMATOGRAM
Retention time: 14.5 (Asp), 15 (Glu), 20 (Asn), 21 (Gln), 22.5 (Ser), 26.5 (Thr, Gly), 27.5 (Tau), 29 (Ala), 32.5 (Arg, Pro), 33 (β-Ala), 36 (Met), 37 (Bal), 39 (Phe), 39.5 (Ile, Leu)

KEY WORDS
derivatization

REFERENCE
Keller,H.J.; Do,K.Q.; Zollinger,M.; Winterhalter,K.H.; Cuénod,M. Cysteine: depolarization-induced release from rat brain in vitro, *J.Neurochem.*, **1989**, *52*, 1801–1806.

SAMPLE
Matrix: perfusate
Sample preparation: Add L-norvaline to a concentration of 750 μM, remove a 1 mL aliquot and add it to 75 μL ice-cold 60% perchloric acid, centrifuge, neutralize supernatant with 200 mM potassium carbonate, add (?) 0.5 M potassium carbonate:potassium hydrogen carbonate 30:70, adjust pH to 9.40-9.50 with 5 M KOH. Remove a 200 μL aliquot and add it to 100 μL 1.25 mg/mL dansyl chloride in MeCN, let stand at room temperature in the dark for 1 h, add 6 μL 0.2% triethylamine in water, add acetic acid to a final concentration of 3%, inject a 20 μL aliquot.

HPLC VARIABLES
Guard column: 30 × 4.6 Biosil ODS-5S microguard refill cartridge (Biorad)
Column: 250 × 4 Biosil C18 ODS-5S (Biorad)
Mobile phase: Gradient. A was MeOH:water 15:85 containing 1% glacial acetic acid and 0.030% triethylamine. B was MeOH:MeCN 70:30 containing 3% glacial acetic acid and

0.030% triethylamine. A:B from 70:30 to 50:50 over 52 min, to 25:75 over 21 min, maintain at 25:75 for 5 min, reset to initial conditions over 7 min.
Flow rate: 1
Injection volume: 20
Detector: F ex 340 em 520

CHROMATOGRAM
Retention time: 10 (Asn), 11 (Gln), 13 (Ser), 14 (Glu), 15 (Asp), 17 (Gly), 18 (Thr), 22 (Ala), 24 (Arg), 32 (Pro), 35 (Met), 37 (Val), 45 (Try), 53 (Phe), 55 (Iso-leu), 57 (Leu), 73 (Orn), 75 (Lys), 79 (His), 83 (Tyr)
Internal standard: L-norvaline (41)

KEY WORDS
rat; derivatization; dansylation

REFERENCE
Zezza,F.; Kerner,J.; Pascale,M.R.; Giannini,R.; Arrigoni Martelli,E. Rapid determination of amino acids by high-performance liquid chromatography: release of amino acids by perfused rat liver, *J.Chromatogr.*, **1992**, *593*, 99–101.

SAMPLE
Matrix: perfusate
Sample preparation: 30 μL Perfusate (artificial CSF) + 10 μL 200 mM perchloric acid. Mix a 25 μL aliquot with 12.5 μL reagent, let stand for 2 min, inject an aliquot. (Prepare a stock solution by dissolving 27 mg o-phthalaldehyde in 1 mL MeOH, add 5 μL β-mercaptoethanol, add 9 mL 100 mM pH 9.3 sodium tetraborate containing 10 μM EDTA. This solution is good for 5 days in a sealed amber bottle at room temperature. Prepare the working reagent by diluting 1 mL of the stock solution with 3 mL 100 mM pH 9.3 sodium tetraborate containing 10 μM EDTA, allow to stand for 24 h before use.)

HPLC VARIABLES
Column: two columns 150 × 4.6 5 μm M.S. Gel C18 (ESA)
Mobile phase: MeCN:MeOH:139 mM Na_2HPO_4 3.1:25:71.9 adjusted to pH 6.8 with phosphoric acid
Column temperature: 33
Flow rate: 1.2
Detector: E, ESA Coulochem Electrode Array System Model 5500, detector temp 33°, oxidation potential 450 mV

CHROMATOGRAM
Retention time: 5.56 (Asp), 6.28 (Glu), 7.50 (Asn), 8.48 (His), 8.76 (Ser), 9.22 (Gln), 10.70 (Arg), 12.75 (Gly), 13.40 (Thr), 18.99 (Tau), 21.69 (Ala), 24.12 (GABA), 25.02 (Tyr)
Limit of detection: 0.75 ng/mL

KEY WORDS
rat; pharmacokinetics

REFERENCE
Acworth,I.N.; Yu,J.; Ryan,E.; Gariepy,K.C.; Gamache,P.; Hull,K.; Maher,T. Simultaneous measurement of monoamine, amino acid, and drug levels, using high performance liquid chromatography and coulometric array technology: application to in vivo microdialysis perfusate analysis, *J.Liq.Chromatogr.*, **1994**, *17*, 685–705.

SAMPLE
Matrix: protein
Sample preparation: Hydrolyze 500 ng protein with 400 μL 6 M HCl at 110° for 24 h (flush tube with argon and evacuate to 0.1 mbar before sealing, alternatively hydrolyze

in liquid phase with 25 μL 6 M HCl), evaporate to dryness under reduced pressure, reconstitute with 20 μL 50 mM pH 8.1 sodium bicarbonate, add 40 μL 4 mM dabsyl chloride in MeCN, heat at 70° for 10 min, dilute to 1 mL with EtOH:50 mM pH 7.0 sodium phosphate 50:50, inject a 20 μL aliquot.

HPLC VARIABLES

Column: 5 μm Lichrosphere 100 CH-18/2
Mobile phase: Gradient. A was DMF:25 mM sodium acetate 4:96, pH 2.5. B was MeCN. A:B from 85:15 to 60:40 over 20 min, to 30:70 over 12 min, maintain at 30:70 for 2 min, return to initial conditions over 2 min, re-equilibrate for 8 min.
Column temperature: 40
Flow rate: 1
Injection volume: 20
Detector: UV 436

CHROMATOGRAM

Retention time: 15 (Aspartate), 16 (Glutamate), 20.5 (Serine), 21 (Threonine), 22 (Glycine), 22.5 (Alanine), 23 (Arginine), 23.5 (Proline), 24 (Valine), 25 (Methionine), 20.5 (Isoleucine), 21 (Leucine), 22 (Phenylalanine), 23.5 (Cysteine), 32 (ammonia), 33.5 (Lysine), 34 (Histidine), 35.5 (Tyrosine)
Limit of detection: <0.5 pmole

KEY WORDS

derivatization

REFERENCE

Knecht,R.; Chang,J.Y. Liquid chromatographic determination of amino acids after gas-phase hydrolysis and derivatization with (dimethylamino)azobenzenesulfonyl chloride, *Anal.Chem.*, **1986**, *58*, 2375–2379.

SAMPLE

Matrix: protein
Sample preparation: Heat 5 mg albumin with 1 mL 6 M HCl, 10 μL mercaptoethanol and 10 μL octanol under vacuum at 110° for 24 h, evaporate to dryness, take up in 1 M HCl, add to a 100 × 5 Dowex 50 ion-exchange column, elute with 1 M HCl, collect fractions. Evaporate fractions to dryness, reconstitute with 2 mL 3% sodium bicarbonate solution, add 4 mL 25 μL/mL fluorodinitrobenzene, let stand in the dark at room temperature overnight, wash with ether, add 2 drops 6 M HCl, extract with ether. Evaporate the ether layer to dryness, reconstitute with EtOH, inject an aliquot.

HPLC VARIABLES

Column: 150 × 4.6 5 μm Ultrasphere ODS
Mobile phase: Gradient. MeOH:30 mM pH 5 KH_2PO_4 from 40:60 to 55:45 over 30 min, maintain at 55:45 for 5 min, return to initial conditions over 5 min, re-equilibrate for 5 min.
Flow rate: 1
Detector: UV 365

CHROMATOGRAM

Retention time: 5 (proline), 10 (tyrosine), 11 (s-methylcysteine), 18 (methionine), 20 (valine), 21 (cystine), 29 (phenylalanine), 31 (isoleucine, leucine)

KEY WORDS

derivatization

REFERENCE

Seago,A.; Shuker,D.E.G.; Paine,A.J. Interaction of [^{14}C]dimethylnitrosamine with albumin produced by rat hepatocytes in culture, *Toxicol.Lett.*, **1986**, *30*, 41–48.

SAMPLE

Matrix: protein

Sample preparation: Grind soybeans to a fine powder, add 6 M HCl, heat at 110° for 22 h, evaporate to dryness in a desiccator over NaOH pellets, reconstitute with EtOH:0.2 N pH 3.2 sodium citrate 7:93, inject an aliquot.

HPLC VARIABLES

Column: 150 × 4 ISC-07/S1504 Na-type (strongly acidic cation-exchange resin of styrene-divinylbenzene copolymer with 10% crosslinkage) (Shimadzu)

Mobile phase: Gradient. A was EtOH:0.2 N pH 3.2 sodium citrate 7:93. B was 0.6 N pH 10.0 sodium citrate. C was 200 mM NaOH. A:B:C from 100:0:0 to 88:12:0 over 20 min, to 40:60:0 (step gradient), to 0:100:0 over 15 min, maintain at 0:100:0 for 5 min, to 0:0:100 (step gradient), maintain at 0:0:100 for 5 min, re-equilibrate at initial conditions for 10 min. (Parameters are approximate.)

Column temperature: 50

Flow rate: 0.3

Detector: F ex 348 em 450 following post-column reaction. The column effluent mixed with the reagent solution pumped at 0.2 mL/min and the mixture flowed through a 200 × 0.5 stainless steel or PTFE coil at 55°. The effluent from the coil mixed with the fluorescence solution pumped at 0.2 mL/min and flowed through a 2 m × 0.5 mm stainless steel or PTFE coil at 55° to the detector. (Prepare reagent solution by adding 400 μL NaOCl solution (chlorine concentration 10%) to 1 L buffer, discard after 2 weeks. Prepare fluorescence solution by adding 15 mL EtOH containing 1.6 g o-phthalaldehyde and 2.0 g N-acetyl-L-cysteine and 4 mL 10% Brij 35 in water to 980 mL buffer, discard after 1 month. Buffer contained 384 mM sodium carbonate, 216 mM boric acid, and 108 mM potassium sulfate, pH 10.0.)

CHROMATOGRAM

Retention time: 8 (Asp), 10 (Thr), 11 (Ser), 13 (Glu), 14 (Pro), 19 (Gly), 20 (Ala), 22 (Cys), 25 (Val), 28 (Met), 30 (Ile), 31 (Leu), 33 (Tyr), 35 (Phe), 40 (His), 45 (Lys), 48 (ammonia), 54 (Arg)

Limit of quantitation: 10 pmole

KEY WORDS

derivatization; post-column reaction; soybeans

REFERENCE

Fujiwara,M.; Ishida,Y.; Nimura,N.; Toyama,A.; Kinoshita,T. Postcolumn fluorometric detection system for liquid chromatographic analysis of amino and imino acids using o-phthalaldehyde/N-acetyl-L-cysteine reagent, *Anal.Biochem.*, **1987**, *166*, 72–78.

SAMPLE

Matrix: protein

Sample preparation: Heat protein in 5.9 M HCl (twice distilled) in a sealed tube at 108° for 22 h, evaporate to dryness under reduced pressure, reconstitute with water, evaporate to dryness under reduced pressure, repeat reconstitution and evaporation step, dry over NaOH under reduced pressure overnight, reconstitute with water. Mix 100 μL of this solution (containing 10-40 μg free amino acids) with 100 μL 500 mM sodium bicarbonate solution and 100 μL freshly prepared 20 mM dansyl chloride, heat at 65° for 40 min (or let stand at room temperature for 24 h) in the dark, inject an aliquot.

HPLC VARIABLES

Column: 250 × 4.6 5 μm Ultrasphere ODS C18

Mobile phase: Gradient. A was MeCN:25 mM NaH_2PO_4 containing 25 mM acetic acid 14:86, adjusted to pH 7.00 with concentrated NaOH. B was MeCN. A:B 100:0 for 5 min, to 92:8 over 5 min, maintain at 92:8 for 15 min, to 55:45 over 30 min, to 42:58 over 5 min, re-equilibrate at initial conditions for 20 min. Every 10-15 runs wash column with 200 mM acetic acid for 40 min.

Flow rate: 1
Injection volume: 20
Detector: UV 254

CHROMATOGRAM

Retention time: 8.49 (Asp), 10.13 (Glu), 16.28 (4-Hydroxyproline), 18.60 (Ser), 19.00 (His (α-derivative)), 19.70 (Arg), 20.14 (Thr), 20.59 (Gly), 22.06 (Ala), 25.18 (Pro), 31.09 (Val), 33.65 (Met), 35.49 (Ile), 35.87 (Leu), 37.85 (Phe), 38.95 (Cys (bis derivative)), 40.85 (ammonia), 44.05 (Desmosine), 44.05 (Isodesmosine), 44.46, 44.80 (5-Hydroxylysine (bis derivative)), 47.34 (Lys (bis derivative)), 49.88 (His (bis derivative)), 52.82 (Tyr (bis derivative))
Limit of quantitation: 100 pmole

KEY WORDS

derivatization

REFERENCE

Negro,A.; Garbisa,S.; Gotte,L.; Spina,M. The use of reverse-phase high-performance liquid chromatography and precolumn derivatization with dansyl chloride for quantitation of specific amino acids in collagen and elastin, *Anal.Biochem.*, **1987**, *160*, 39–46.

SAMPLE

Matrix: protein
Sample preparation: 0.1-1 μg protein + 20 μL 5.7 M HCl (twice distilled), seal under reduced pressure, heat at 110° for 18 h, dry under vacuum, add 10 μL 4 mM dabsyl chloride in acetone, heat at 70° for 10 min, dry under vacuum, reconstitute with 100-500 μL MeCN:water 50:50 over EtOH:water 70:30, inject a 10 μL aliquot.

HPLC VARIABLES

Column: 150 × 4.6 Cosmosil 5 octadecylsilane (Nakarai Chemicals, Kyoto)
Mobile phase: Gradient. A was DMF:17 mM pH 6.5 phosphate buffer 2:98. B was MeCN:DMF 96:4. A:B from 85:15 to 55:45 over 25 min, to 30:70 over 10 min.
Flow rate: 1
Injection volume: 10
Detector: UV 420

CHROMATOGRAM

Retention time: 13.5 (Asp), 14.5 (Glu), 20 (Ser), 20.8 (Thr), 21.5 (Gly), 21.8 (Ala), 22.2 (Arg), 22.5 (Pro), 23 (Val), 24.5 (Met), 25 (Ile), 25.5 (Leu), 27 (Phe), 35 (Lys), 35.5 (His), 38 (Tyr)

KEY WORDS

derivatization

REFERENCE

Odani,S.; Kenmochi,N.; Ogata,K. A comparative study on 40S ribosomal proteins of Artemia salina and rat liver: micro analysis of amino acid composition by high-performance liquid chromatography, *J.Biochem.(Tokyo)*, **1988**, *103*, 872–877.

SAMPLE

Matrix: protein
Sample preparation: Hydrolyze 50-100 pmole protein with 200 μL 6 M HCl in a sealed argon-flushed vial at 110° for 24 h, evaporate to dryness, add 50 μL triethylamine:water 25:75, evaporate to dryness under reduced pressure, repeat this step, add 100 μL triethylamine:water 25:75, add 100 μL 1 mM Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) in acetone, mix, shake gently in the dark at 40° for 1 h, add 20 μL 2 M HCl, dry under reduced pressure, dissolve in DMSO:water 50:50, inject an aliquot.

HPLC VARIABLES

Guard column: 20 × 4.6 5 μm Nucleosil C8
Column: 250 × 4.6 5 μm Aquapore RP-300 C8
Mobile phase: Gradient. A was THF:13 mM trifluoroacetic acid 4:96. B was MeCN:THF: 13 mM trifluoroacetic acid 50:2:48. A:B from 100:0 to 65:35 over 50 min, to 60:40 (step gradient), maintain at 60:40 for 5 min, to 15:85 over 55 min.
Flow rate: 1
Injection volume: 50
Detector: UV 340

CHROMATOGRAM

Retention time: 22.5 (cysteic acid), 35.5 (S-carboxymethylcysteine), 40 (serine), 41.5 (threonine), 43.5 (arginine), 46 (glycine), 48 (aspartic acid), 54 (glutamic acid), 54.5 (alanine), 56 (proline), 68 (histidine), 68.5 (methionine), 70 (valine), 78.5 (isoleucine), 80.5 (leucine), 82.5 (tryptophan), 84 (phenylalanine), 87 (lysine), 88.5 (cysteine), 104.5 (tyrosine)
Limit of detection: 50 pmole

KEY WORDS

derivatization; comparison with other derivatizing reagents

REFERENCE

Kochhar,S.; Christen,P. Amino acid analysis by high-performance liquid chromatography after derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, *Anal.Biochem.*, **1989**, *178*, 17–21.

SAMPLE

Matrix: protein
Sample preparation: Add 200 μL concentrated HCL containing 1% phenol to 0.1-5 μg protein, seal tube under vacuum, heat at 150° for 1 h or 108° for 24 h, evaporate to dryness under reduced pressure, add 10-20 μL EtOH:water:triethylamine 40:40:20, evaporate to dryness under reduced pressure, add 20 μL reagent, let stand at room temperature for 20 min, evaporate to dryness under reduced pressure, reconstitute with PTC amino acid diluent (Waters), inject a 1-40 μL aliquot (J.Chromatogr. 1984, 336, 93). (Reagent was EtOH:triethylamine:water:phenylisothiocyanate 70:10:10:10, store at -20°.)

HPLC VARIABLES

Column: 150 × 3.9 Nova Pak C18
Mobile phase: Gradient. A was MeCN:145 mM pH 6.2 ammonium acetate 6:94. B was MeCN:water 60:40. A:B from 100:0 to 40:60 over 15 min (Waters convex gradient 5), clean with 0:100 for 2 min.
Column temperature: 43
Flow rate: 0.7
Injection volume: 1-40
Detector: UV 254 or MS, VG Masslab Model 30-250 quadrupole, VG Masslab thermospray interface, the column effluent was combined with 200 mM ammonium acetate pumped at 0.3 mL/min and this mixture flowed into the MS, thermospray nozzle 32°, thermospray chamber 340

CHROMATOGRAM

Retention time: 1.6 (Asp), 1.9 (Glu), 4.7 (Ser), 5.0 (Gly), 6.1 (His), 8.1 (Arg), 8.7 (Thr), 9.3 (Ala), 10.1 (ammonia), 11.2 (Pro), 14.5 (Tyr), 15.4 (Val), 16.0 (Met), 16.8 (Cys), 17.3 (Ile), 17.5 (Leu), 18.6 (Phe), 20.2 (Lys)

KEY WORDS

derivatization

REFERENCE

Pramanik,B.C.; Moomaw,C.R.; Evans,C.T.; Cohen,S.A.; Slaughter,C.A. Identification of phenylthiocarbamyl amino acids for compositional analysis by thermospray liquid chromatography/mass spectrometry, *Anal.Biochem.*, **1989**, *176*, 269–277.

SAMPLE

Matrix: protein

Sample preparation: Treat 10 nmoles protein immobilized on 5 mg p-phenylenediisothiocyanate glass with 20 mg thio-acetylated glass beads (Sigma), 500 μL 10% thioacetylthioglycolic acid in MeOH, and 200 μL 20% triethylamine in MeOH with constant agitation at 45° for 30 min, wash with 2 mL 20% triethylamine in MeOH, wash with 3 mL MeOH, wash with 3 mL dichloromethane, dry under vacuum for 15 min, add 500 μL trifluoroacetic acid, let stand at room temperature for 15 min. Remove the acid, wash the solid three times with 200 μL portions of dichloromethane. Combine the acid and the washes and add 100 μL 4-nitrobenzenesulfonyl chloride (2.5 eq) in dichloromethane. Evaporate to dryness under a stream of nitrogen at 35°, reconstitute the residue in 500 μL dichloromethane, add 50 μL 20% triethylamine in dichloromethane, concentrate immediately under reduced pressure, reconstitute, inject a 20 μL aliquot. (Synthesis of thioacetylthioglycolic acid is as follows. Reflux 8.8 g acetaldehyde, 30 mL piperidine, and 9.6 g sulfur for 1 h, cool, add water, acidify with concentrated HCl, crush the solid, wash with water, recrystallize from 200 mL EtOH to give thioacetylpiperidine (mp 55-56°). Dissolve 10 g thioacetylpiperidine in 50 mL dry benzene (Caution! Benzene is a carcinogen!), add 10.7 g bromoacetic acid, let stand at room temperature for 24 h, add 150 mL dry ether, recrystallize from EtOH/ether to give S-carboxyethylthiomethylpiperidinium bromide (mp 168-169°). Dissolve 10.1 g S-carboxyethylthiomethylpiperidinium bromide in 40 mL EtOH, cool in ice, pass hydrogen sulfide through the solution at 2-3 bubbles/s for 3-4 h (Caution! Hydrogen sulfide is highly toxic!), let stand overnight at 0°, remove EtOH by evaporation under reduced pressure, repeatedly extract the residue with ether until it is colorless. Evaporate the ether solution to dryness under reduced pressure, recrystallize from light petroleum (bp 60-100°) to obtain thioacetylthioglycolic acid (mp 80-81°) (Acta Chem. Scand. 1961, 15, 1087). 4-Acetylmorpholine can also be used as the starting reagent (Anal. Biochem. 1989, 181, 113).)

HPLC VARIABLES

Column: 150 × 4.6 5 μm IBM C18

Mobile phase: Gradient. MeCN:MeOH:buffer from 35:0:65 to 42:0:58 over 4.9 min, to 36:9:55 over 0.1 min, to 60:15:25 over 10 min, maintain at 60:15:25 for 2 min, to 35:0:65 over 0.1 min, maintain at 35:0:65 for 3.9 min. (Prepare buffer by dissolving 10 mL acetic acid, 4 mL triethylamine, and 2 mL trifluoroacetic acid, pH 3.1.)

Flow rate: 1.5

Injection volume: 20

Detector: UV 248

CHROMATOGRAM

Retention time: 3.2 (Asn), 3.7 (Gln), 4.6 (Asp), 5.0 (Arg), 5.9 (Glu), 8.3 (Gly), 9.3 (Ala), 10.0 (Lys), 12.6 (Met), 13.4 (Val), 13.9 (Trp), 14.7 (Phe), 15.0 (Leu, Ile), 17.0 (Tyr)

KEY WORDS

derivatization

REFERENCE

Stolowitz,M.L.; Paape,B.A.; Dixit,V.M. Thioacetylation method of protein sequencing: derivatization of 2-methyl-5(4*H*)-thiazolones for high-performance liquid chromatographic detection, *Anal.Biochem.*, **1989**, *181*, 113–119.

SAMPLE

Matrix: protein

Sample preparation: Hydrolyze protein with 80 μL 3% phenol in 6 M HCl in an evacuated tube at 166° for 25 min, evaporate to dryness under reduced pressure at 50°, reconstitute with 10 μL 200 mM pH 9.0 carbonate buffer, add 20 μL 4 mM dabsyl chloride in MeCN, heat at 70° with occasional shaking for 10 min, dilute to 200 μL with MeCN:water 66:34, centrifuge, inject a 10 μL aliquot of the supernatant.

HPLC VARIABLES

Column: 100 × 4.6 3 μm Hypersil ODS
Mobile phase: Gradient. A was acetone:10 mM pH 6.5 sodium phosphate buffer 7.5:92.5. B was acetone:10 mM pH 6.5 sodium phosphate buffer 50:50. A:B from 100:0 to 0:100 over 40 min. (Place a 50 × 4.6 column of 30 μm Wakogel LC ODS-30K before the injector.)
Column temperature: 60
Flow rate: 1
Injection volume: 10
Detector: UV 436

CHROMATOGRAM

Retention time: 11 (aspartic acid), 12 (glutamic acid), 18.5 (serine), 19.3 (threonine), 19.7 (glycine), 20.5 (alanine), 21.5 (proline), 23 (valine), 24 (arginine), 25 (methionine), 26 (isoleucine), 26.5 (leucine), 27 (tryptophan), 27.5 (phenylalanine), 30 (cysteine), 38.5 (lysine), 39.5 (histidine), 43 (tyrosine)

KEY WORDS

derivatization

REFERENCE

Muramoto,K.; Kamiya,H. Recovery of tryptophan in peptides and proteins by high-temperature and short-term acid hydrolysis in the presence of phenol, *Anal.Biochem.*, **1990**, *189*, 223–230.

SAMPLE

Matrix: protein
Sample preparation: Dissolve 1 mg protein in 2 mL 6 M HCl, heat in a sealed tube at 90° for 12 h, evaporate to dryness under reduced pressure, reconstitute with 2 mL water. Mix 25 μL hydrolyzate, 25 μL pH 9.1 saturated sodium borate, 25 μL 20 mM cetyltrimethylammonium bromide, 12.5 μL MeCN, and 10 mg polymeric reagent, heat at 70° for 10 min, elute with 1 mL MeCN:water 70:30, inject a 20 μL aliquot of the eluate. (Prepare polymeric reagent as follows. Soxhlet extract styrene-divinylbenzene copolymer (12% cross-linked, 60 Å templated, 10-20 μm, Supelco) with dioxane for 8 h (Caution! Dioxane is a carcinogen!). Add 25 g aluminum trichloride in 300 mL dry nitrobenzene to 50 g resin and 100 g 4-chloro-3-nitrobenzoyl chloride, stir mechanically at 60° for 5 h, pour into a mixture of 150 mL DMF, 100 mL concentrated HCl, and 150 g ice, filter. Wash the solid with 300 mL portions of DMF:water 75:25 until the washings are colorless, wash with warm (60°) DMF, wash with six 300 mL portions of dichloromethane:MeOH 2:1. Stir the product in 130 mL 40% benzyltrimethylammonium hydroxide in water, 130 mL water, and 260 mL dioxane at 90° for 8 h, filter, repeat the process. Wash the product with four portions of warm (60°) dioxane. Stir the solid with 30 mL acetic acid for 15 min, filter. Wash the solid with dioxane until the washings are neutral, wash with six 300 mL portions of dichloromethane:MeOH 2:1 to give a nitrobenzophenol-substituted polymer (J. Org. Chem. 1984, 49, 924). Heat 4 g 9-fluoreneacetic acid, 3.9 mL oxalyl chloride, 30 mL benzene (dried over anhydrous sodium sulfate, Caution! Benzene is a carcinogen!), and 3 drops of triethylamine at 55° for 1 h, evaporate under reduced pressure to remove oxalyl chloride, dissolve the product in 35 mL dichloromethane to give a 120 mg/mL solution of 9-fluoreneacetyl chloride, dilute to obtain a 2 mM solution. Stir 1.3 g nitrobenzophenol-substituted polymer, 4.2 mL 2 mM 9-fluoreneacetyl chloride solution, 300 μL triethylamine, and 20 mL dichloromethane at room temperature for 1 h, filter, wash with three 20 mL portions of MeCN to obtain the reagent, polymer-bound nitrobenzophenol 9-fluoreneacetate (J. Chromatogr. 1992, 609, 103).)

HPLC VARIABLES
Column: 250 × 4.6 YMC AP-303 300 Å ODS (YMC)
Mobile phase: Gradient. A was 0.05% trifluoroacetic acid in water. B was 0.05% trifluoroacetic acid in MeCN. A:B from 70:30 to 30:70 over 16 min, maintain at 30:70 for 6 min, return to initial conditions over 30 s.
Flow rate: 1.5
Injection volume: 20
Detector: F ex 254 em 305-395

CHROMATOGRAM
Retention time: 5.8 (His), 6.3 (Asn, Gln), 7.2 (Ser), 7.4 (Glu), 7.8 (Thr), 9.1 (Ala), 10.6 (Pro), 11 (Met), 12.2 (Tyr), 12.6 (Ile), 12.8 (Leu), 13 (Phe), 14.6 (Lys)

KEY WORDS
derivatization

REFERENCE
Zhou,F.-X.; Krull,I.S.; Feibush,B. Solid-phase derivatization of amino acids and peptides in high-performance liquid chromatography, *J.Chromatogr.*, **1993**, *648*, 357–365.

SAMPLE
Matrix: protein
Sample preparation: Heat 50 μg protein with 200 μL 6 M HCl and 1 crystal of phenol at 153° for 1 h, evaporate to dryness under reduced pressure, reconstitute with water. Mix 25 μL hydrolyzate, 25 μL saturated sodium borate, 25 μL 20 mM cetyltrimethylammonium bromide, 12.5 μL MeCN, and 15 mg polymeric 6-aminoquinoline reagent, heat at 70° for 10 min, elute with 1 mL MeCN:water 70:30, inject a 20 μL aliquot of the eluate. (Prepare polymeric 6-aminoquinoline reagent as follows. React 10 g 4-chloro-3-nitrobenzyl alcohol, 10 g dried 16-20 μm styrene-divinylbenzene copolymer (12% cross-linked, 102 Å templated, Supelco), and 10 g anhydrous aluminum chloride in 50 mL nitrobenzene at 65-70° for 3 days, cool to room temperature, filter, wash with three 50 mL portions of 1 M HCl in dioxane (Caution! Dioxane is a carcinogen!), wash with three 50 mL portions of DMF, wash with three 50 mL portions of MeOH, wash with three 50 mL portions of dichloromethane, dry under reduced pressure at 100°. Reflux 19 g of this polymer in 60 mL hydrazine hydrate:2-ethoxyethanol 40:60 for 20 h, cool to room temperature, filter, wash thoroughly with water, suspend the polymer in 100 mL concentrated aqueous HCl: dioxane 50:50, reflux for 20 h, filter, wash with five 100 mL portions of water, wash with three 100 mL portions of MeOH, wash with three 50 mL portions of ether, dry under reduced pressure at 80° to obtain polymeric benzotriazole reagent (Eur. J. Biochem. 1975, 59, 55). Heat 1.6 g quinoline-6-carboxylic acid (ICN Biomedicals, Costa Mesa CA) with 2.7 mL thionyl chloride in 55 mL dry benzene at 65-75° for 1 h (Caution! Benzene is a carcinogen!), remove the benzene by evaporation under reduced pressure, add 21 mL acetic acid, stir strongly for several min, slowly add 600 mg sodium azide at room temperature, stir for 1 h, add 53 mL triethylamine, add 100 mL water, stir for 30 min, filter, wash ten times with 25 mL portions of water, dry under vacuum overnight to obtain quinoline-6-acyl azide. Heat 3.3 g polymeric benzotriazole reagent and 2 g quinoline-6-acyl azide in 150 mL toluene (dried over calcium hydride) at 60-65° for 2 h, wash with 600 mL warm (40°) dichloromethane, wash with 50 mL MeCN, dry under vacuum overnight to obtain polymeric 6-aminoquinoline reagent.)

HPLC VARIABLES
Column: 150 × 3.9 4 μm Nova-Pak C18
Mobile phase: Gradient. A was 140 mM sodium acetate containing 17 mM triethylamine, adjusted to pH 5.05 with phosphoric acid. B was MeCN:water 60:40. A:B from 100:0 to 50:50 over 30 min, to 0:100 (step gradient), maintain at 0:100 for 5 min, return to initial conditions, re-equilibrate at initial conditions for 10 min.
Flow rate: 1.5
Injection volume: 20

Detector: F ex 254 em 395

CHROMATOGRAM
Retention time: 15-30 (depending on structure, peaks not identified)
Limit of detection: 1 ppm

KEY WORDS
derivatization

REFERENCE
Yu,J.H.; Li,G.D.; Krull,I.S.; Cohen,S. Polymeric 6-aminoquinoline, an activated carbamate reagent for derivatization of amines and amino acids by high-performance liquid chromatography, *J.Chromatogr.B*, **1994**, *658*, 249–260.

SAMPLE
Matrix: protein
Sample preparation: Heat 300 mg protein and 1.5 mL 6 M HCl at 110° in a sealed tube for 24 h, evaporate to dryness. Dissolve an amount of protein hydrolysate containing 4 mmole amino acids in 2 mL 2 M NaOH, stir at 0°, add 1.36 g benzyl chloroformate and 250 μL 4 M NaOH simultaneously over 40 min so as to maintain the pH between 10 and 12, wash the reaction mixture with 250 μL pentane. Cool the aqueous layer and acidify to Congo red with 4 M HCl, extract with three 200 μL portions of ethyl acetate. Combine the extracts and dry them over anhydrous sodium sulfate, evaporate to dryness under reduced pressure, reconstitute with MeOH, inject a 10 μL MeOH.

HPLC VARIABLES
Column: 250 × 4.6 5 μm Econosil C18
Mobile phase: Gradient. A was 0.5% trifluoroacetic acid in water. B was 0.5% trifluoroacetic acid in MeCN. A:B from 100:0 to 40:60 over 1 h, to 0:100 over 5 min, maintain at 0:100 for 5 min, return to initial conditions over 5 min, re-equilibrate for 10 min.
Flow rate: 1.5
Injection volume: 10
Detector: UV 254

CHROMATOGRAM
Retention time: 17 (Asn), 18 (Gln), 18.5 (His), 19 (Ser), 20 (Thr), 22 (Asp), 23 (Glu), 24 (Gly), 28 (Ala), 33 (Pro), 34 (Tyr), 39 (Val), 42 (Met), 45.5 (Leu), 45.5 (Ile), 45.5 (Trp), 46 (Phe), 49.5 (Lys), 56 (Cys), 58 (Arg)

KEY WORDS
derivatization

REFERENCE
Egorova,T.A.; Eremin,S.V.; Mitsner,B.I.; Zvonkova,E.N.; Shvets,V.I. Isolation of individual amino acids from various microbiological sources using reversed-phase high-performance liquid chromatography, *J.Chromatogr.B*, **1995**, *665*, 53–62.

SAMPLE
Matrix: protein
Sample preparation: Heat 2 mg protein and 1 mL 6 M HCl in a sealed tube at 110° for 20 h, reconstitute with 5 mL buffer. Remove a 400 μL aliquot and add it to 600 μL buffer and 400 μL 2 mM 9-isothiocyanatoacridine in MeCN, heat at 60° with occasional shaking for 1.5 h, cool, inject a 20 μL aliquot. (Prepare buffer by mixing 500 mL 100 mM boric acid containing 100 mM KCl with 408 mL 100 mM NaOH, make up to 1 L with water, pH 9.8. Prepare 9-isothiocyanatoacridine as follows. Reflux 15 mmoles 9-chloroacridine (Eastman) and 16 mmoles silver thiocyanate (Aldrich) in 150 mL anhydrous toluene, recrystallize 9-isothiocyanatoacridine from anhydrous acetone, mp 131-2° (Chem.Abs. 1970, 72, 21584v).)

HPLC VARIABLES
Column: 150 × 3.3 5 μm Separon C-18 SGX (Tessek, Prague)
Mobile phase: MeCN:23 mM pH 6 ammonium formate 20:80
Flow rate: 0.2
Injection volume: 20
Detector: UV 280

CHROMATOGRAM
Retention time: 4.62 (Glu), 4.85 (Asp), 5.88 (Ala), 6.44 (Asn), 6.83 (Gly), 7.10 (Ser), 7.90 (Thr), 8.37 (His), 10.73 (Gln), 11.63 (Val), 12.27 (Tyr), 13.37 (Pro, Lys), 15.20 (Arg), 16.97 (Met), 26.07 (Ile), 26.60 (Leu), 36.93 (Phe), 38.97 (Trp)
Limit of detection: 100-400 pmole

KEY WORDS
derivatization

REFERENCE
Oravec,P.; Podhradsky,D. High-performance liquid chromatography of amino acids after derivatization with 9-isothiocyanatoacridine, *J.Biochem.Biophys.Methods*, **1995**, *30*, 145–152.

SAMPLE
Matrix: protein
Sample preparation: For N-terminal amino acid. Add 200 pmole protein and 200 pmole nor-leucine to a tube, dry under reduced pressure, dissolve in 40 μL pH 9.5 lithium carbonate buffer, add 20 μL 1.5 mg/mL dansyl chloride in MeCN, shake for 2 min, heat at 37° for 40 min, add 2 μL 8.9 M ethylamine hydrochloride, heat at 37° for 10 min, evaporate to dryness under reduced pressure at room temperature, reconstitute with 100 μL constant boiling HCl, heat at 110° for 18 h, evaporate to dryness over solid NaOH, reconstitute with 100 μL initial mobile phase, inject an aliquot. For amino acid mixtures. Add 20 nmole amino acid mixture to a tube, dry under reduced pressure, dissolve in 40 μL pH 9.5 lithium carbonate buffer, add 20 μL 1.5 mg/mL dansyl chloride in MeCN, shake for 2 min, heat at 37° for 40 min, add 2 μL 8.9 M ethylamine hydrochloride, heat at 37° for 10 min, evaporate to dryness under reduced pressure at room temperature, reconstitute with 100 μL initial mobile phase, inject an aliquot.

HPLC VARIABLES
Guard column: 50 mm long 37-50 μm C18/Corasil
Column: 150 × 3.9 4 μm NovaPak C18
Mobile phase: Gradient. A was MeOH:THF:100 mM pH 7.4 sodium phosphate buffer 5:6.5:88.5. B was MeOH:water 70:30. A:B from 100:0 to 0:100 over 30 min (Waters curve no.6), return to initial conditions over 10 min, re-equilibrate at initial conditions for 10 min. (At the end of each day wash column with 35 mL water and 20 mL MeOH:water 70:30.)
Column temperature: 25
Flow rate: 1
Detector: F ex 338 (bandpass filter) ex 455 (long-pass filter)

CHROMATOGRAM
Retention time: 4.5 (Asp), 5.5 (Glu), 12 (Ser), 12.5 (Arg), 13 (Thr), 14 (Gly), 14.7 (Ala), 15 (Pro), 18.5 (Val), 19 (ammonia), 20 (Met), 21.5 (Ile), 22 (Leu), 23.5 (Phe), 25 (ethylamine), 26 (cystine, bis-derivative), 29 (Lys, bis-derivative), 30.5 (His, bis-derivative), 31 (Tyr, bis-derivative)
Internal standard: norleucine (23)
Limit of detection: 2 pmole

KEY WORDS
derivatization

REFERENCE

Martins,A.R.; Padovan,A.P. A practical approach to improve the resolution of dansyl-amino acids by high-performance liquid chromatography, *J.Liq.Chromatogr.Rel.Technol.*, **1996**, *19*, 467–476.

SAMPLE

Matrix: protein

Sample preparation: Hydrolyze protein with 1 mL 6 M HCl for each 1 mg protein, heat under nitrogen at 110° for 24 h, filter (paper), make up the filtrate to 250 mL, evaporate to dryness under reduced pressure at 40°, reconstitute the residue with water so as to give a protein concentration of 150-250 μg/mL. 1 mL Hydrolysate + 2 mL 40 mM pH 9.5 lithium carbonate + 1 mL 4 mg/mL dansyl chloride in MeCN, mix, heat at 60° for 30 min, add 50 μL 2% methylamine in water, inject an aliquot.

HPLC VARIABLES

Column: 300 × 3.9 10 μm Spherisorb ODS-2
Mobile phase: MeCN:10 mM pH 7.0 phosphate buffer 39:61
Column temperature: 40
Flow rate: 1.5
Injection volume: 20
Detector: UV 254

CHROMATOGRAM

Retention time: 4.2 (lysine, bis derivative), 4.7 (histidine, bis derivative), 6.7 (ammonia), 11.7 (tyrosine), 15 (methylamine)

KEY WORDS

casein; lysozyme; lentils; enteral solution; derivatization

REFERENCE

Sanz,M.A.; Castillo,G.; Hernández,A. Isocratic high-performance liquid chromatographic method for quantitative determination of lysine, histidine and tyrosine in foods, *J.Chromatogr.A*, **1996**, *719*, 195–201.

SAMPLE

Matrix: protein hydrolysate

Sample preparation: 2-200 μg Protein hydrolysate + 1 mL 1 M pH 9.0 sodium borate buffer containing 0.02% sodium azide + 0.8 μL diethyl ethoxymethylenemalonate (Fluka), heat at 50° with vigorous shaking for 50 min, cool to room temperature, inject a 15 μL aliquot.

HPLC VARIABLES

Column: 300 × 3.9 4 μm Nova-Pak C18

Mobile phase: Gradient. MeCN:buffer from 9:91 to 14:86 over 3 min, maintain at 14:86 for 10 min, to 31:69 over 17 min, maintain at 31:69 for 5 min. (Buffer was 25 mM pH 6.0 sodium acetate containing 0.02% sodium azide. Caution! Sodium azide is highly toxic! Do not discharge solutions containing sodium azide to the plumbing system!)

Column temperature: 18
Flow rate: 0.9
Injection volume: 15
Detector: UV 280

CHROMATOGRAM

Retention time: 3.10 (aspartic acid), 3.31 (glutamic acid), 7.48 (serine), 9.35 (histidine), 10.25 (glycine), 11.02 (threonine), 12.66 (arginine), 13.45 (alanine), 15.25 (proline), 20.05 (tyrosine), 24.21 (ammonia), 25.84 (valine), 27.22 (methionine), 28.52 (cystine), 30.19 (isoleucine), 31.02 (leucine), 31.95 (phenylalanine), 34.73 (lysine)

Internal standard: α-aminobutyric acid (18.15)

Limit of detection: 3 pmole

KEY WORDS
derivatization

REFERENCE
Alaiz,M.; Navarro,J.L.; Girón,J.; Vioque,E. Amino acid analysis by high-performance liquid chromatography after derivatization with diethyl ethoxymethylenemalonate, *J.Chromatogr.*, **1992**, *591*, 181–186.

SAMPLE
Matrix: protein hydrolysate, exopeptidase digest
Sample preparation: 5-10 μL Protein hydrolysate or exopeptidase digest + 40 μL MeOH:triethylamine 8:1 + 5 μL phenylisothiocyanate, mix, let stand at room temperature for 20 min, wash twice with 15 μL portions of heptane, dilute the aqueous phase with 40 μL 50 mM pH 5.4 sodium acetate buffer:acetic acid 100:3, let stand for 30 s, add 40 μL 50 mM pH 5.4 sodium acetate buffer:acetic acid 100:3, let stand for 30 s, inject a 90 μL aliquot.

HPLC VARIABLES
Mobile phase: Gradient. A was 50 mM pH 5.4 sodium acetate. B was MeCN:water 70:30. A:B from 93:7 to 70:30 over 10 min, to 42:58 over 10 min.
Column temperature: 37
Flow rate: 0.3
Injection volume: 90
Detector: UV 254

CHROMATOGRAM
Retention time: 5.5 (Asp), 6 (Glu), 6.7 (Ser), 7.2 (Gly), 7.5 (His), 8 (Arg), 8.5 (Thr), 9 (Ala), 9.3 (Pro), 11.6 (Tyr), 12.6 (Val), 13 (Met), 13.7 (Cystine), 14.8 (Ile), 15 (Leu), 15.4 (Norleucine), 15.8 (Phe), 17 (Lys)
Limit of quantitation: 50 pmole

KEY WORDS
derivatization

REFERENCE
Thoma,R.S.; Crimmins,D.L. Automated phenylthiocarbamyl amino acid analysis of carboxypeptidase/aminopeptidase digests and acid hydrolysates, *J.Chromatogr.*, **1991**, *537*, 153–165.

SAMPLE
Matrix: shrimp
Sample preparation: Blend (Waring) 1 g shrimp and 2 mL trichloroacetic acid, centrifuge, mix an aliquot of the supernatant with equal volumes of 400 mM borate buffer and 2 mg/mL 4-chloro-7-nitrobenzofurazan in MeOH, heat at 60° for 5 min, inject a 10 μL aliquot.

HPLC VARIABLES
Column: 300 × 4.6 5 μm Lichrosorb RP-C18
Mobile phase: Gradient. A was THF:100 mM sodium acetate buffer 1:99, pH 6.2. B was MeOH. A:B from 80:20 to 70:30 over 5 min, maintain at 70:30 for 3 min, to 50:50 over 1.5 min, maintain at 50:50 for 3 min, return to initial conditions over 2 min.
Flow rate: 1.2
Injection volume: 10
Detector: F ex 220 em 370 (filter)

CHROMATOGRAM
Retention time: 4.5 (hydroxyproline), 8.9 (proline)

KEY WORDS
derivatization

REFERENCE
Vázquez-Ortiz,F.A.; Caire,G.; Higuera-Ciapara,I.; Hernández,G. High performance liquid chromatographic determination of free amino acids in shrimp, *J.Liq.Chromatogr.*, **1995**, *18*, 2059–2068.

SAMPLE
Matrix: solutions

HPLC VARIABLES
Column: 250 × 4.6 6μm Zorbax TMS
Mobile phase: 1.8 mM pH 4.1 copper sulphate (Prepare mobile phase by dissolving 450 mg copper sulfate pentahydrate in 1 L water and adjusting the pH to 4.1 with 50 mM sulfuric acid.)
Flow rate: 1
Injection volume: 10
Detector: UV 230

CHROMATOGRAM
Retention time: 3.517 (glutamic acid), 4.142 (threonine), 5.007 (ornithine), 5.812 (histidine), 6.887 (arginine), 10.425 (phenylalanine)

REFERENCE
Khedr,A. High-performance liquid chromatography of α-amino acids and aztreonam on reversed phase columns with aqueous Cu2+ as eluent, *Biomed.Chromatogr.*, **1996**, *10*, 167–171.

SAMPLE
Matrix: solutions
Sample preparation: 20-100 μL Amino acid solution in water + 20-100 μL pH 9.0 Titrisol buffer (Merck) + 20-100 μL 2.7 mg/mL dansyl chloride in water-free acetone, let stand in the dark at room temperature for 1 h, evaporate to dryness under reduced pressure, reconstitute with 100 μL acetone:1 M HCl 95:5, inject a 0.1-1 μL aliquot.

HPLC VARIABLES
Column: 500 × 3 5 μm LiChrosorb SI 60
Mobile phase: Gradient. A was benzene:pyridine:acetic acid 100:10:1. B was pyridine:acetic acid 90:9. A:B from 100:0 to 0:100 over 25 min in a complex fashion. The gradient was obtained by adding 15 mL B over 15 min to a reservoir containing 50 mL A, then adding 15 mL B over 5 min. While these additions were happening the mobile phase was pumped out of the reservoir at 1 mL/min. Re-equilibrate at initial conditions for 5 min before the next injection. (Caution! Benzene is a carcinogen!)
Column temperature: 65
Flow rate: 1
Injection volume: 0.1-1
Detector: F ex 340 em 510

CHROMATOGRAM
Retention time: 4 (isoleucine), 5 (leucine), 6 (valine), 7.5 (proline), 10 (phenylalanine), 12 (methionine), 13 (alanine), 14 (lysine, didansyl), 15 (tyrosine), 17 (glycine), 18 (tryptophan), 19 (glutamic acid), 20 (threonine), 22 (serine), 23 (aspartic acid), 2.5 (cystine, mobile phase benzene:pyridine:acetic acid:MeOH 100:50:0.5:50), 3 (histidine, mobile phase benzene:pyridine:acetic acid:MeOH 100:50:0.5:50), 5 (arginine, mobile phase benzene:pyridine:acetic acid:MeOH 100:50:0.5:50)

KEY WORDS
derivatization; normal phase

REFERENCE

Bayer,E.; Grom,E.; Kaltenegger,B.; Uhmann,R. Separation of amino acids by high performance liquid chromatography, *Anal.Chem.*, **1976**, *48*, 1106–1109.

SAMPLE

Matrix: solutions

Sample preparation: Mix a 50 μL aliquot of a 1 mg/mL amino acid solution in MeCN: triethylamine 99.8:0.2 with 50 μL 0.5% 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate in MeCN, let stand at room temperature for 1 h, inject a 5 μL aliquot. (2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate may be purchased from Aldrich, synthesis details are also given in this paper.)

HPLC VARIABLES

Column: 250 × 4 5 μm LiChrosorb RP-18

Mobile phase: MeOH:water 50:50 (A) or 60:40 (B)

Flow rate: 0.4

Injection volume: 5

Detector: UV 250

CHROMATOGRAM

Retention time: 10 (D-Ser (A)), 11 (L-Ser (A)), 14 (L-Tyr (B)), 15 (L-Ala (A)), 16 (D-Ala (A), D-Tyr (B)), 19 (L-Val (B)), 21 (D-Val (B)), 22 (L-phenylglycine (B)), 23 (D-phenylglycine (B)), 25 (L-Asp (A)), 28 (L-Leu (B)), 28.5 (D-Asp (A), L-Glu (A)), 30 (D-Glu (A)), 32 (D-Leu (B), L-Phe (B)), 38 (D-Phe (B))

Limit of detection: 5 ng

KEY WORDS

derivatization; chiral

REFERENCE

Nimura,N.; Ogura,H.; Kinoshita,T. Reversed-phase liquid chromatographic resolution of amino acid enantiomers by derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate, *J.Chromatogr.*, **1980**, *202*, 375–379.

SAMPLE

Matrix: solutions

Sample preparation: Rapidly mix 2 mL 0.001-1 mM amino acid solution in buffer with 1 mL 1.5 mg/mL dansyl chloride in MeCN, shake gently for 2 min, let stand at room temperature for 35 min, add 100 μL 2% ethylamine hydrochloride, inject a 25 μL aliquot. (Buffer was 40 mM lithium carbonate adjusted to pH 9.5 with HCl. Distil MeCN from dansyl chloride to remove trace impurities. Protect reaction from light.)

HPLC VARIABLES

Column: 150 × 4.6 5 μm Supelcosil C8

Mobile phase: MeOH:buffer 42:58 (Buffer was 0.6% glacial acetic acid containing 0.008% triethylamine.)

Flow rate: 2

Injection volume: 25

Detector: F ex 250 em 470

CHROMATOGRAM

Retention time: 2.5 (Asn), 3.5 (ammonia), 4 (Asp), 5.5 (Thr), 7 (Ala), 17 (Met)

Limit of detection: 1 pmole

KEY WORDS

derivatization

REFERENCE

Tapuhi,Y.; Schmidt,D.E.; Lindner,W.; Karger,B.L. Dansylation of amino acids for high-performance liquid chromatography analysis, *Anal.Biochem.*, **1981**, *115*, 123–129.

SAMPLE

Matrix: solutions

Sample preparation: 1 mL Solution + 3 mL 200 mM pH 9.0 borate buffer + 1 mL 30 mM 2,4,6-trinitrobenzenesulfonic acid in water, let stand at room temperature for 1 h, dilute, inject an aliquot. Alternatively, acidify reaction mixture with 1 drop 6 M HCl, add 2.5 mL hexane:ethyl acetate 20:80, vortex for 15 s. Remove 2 mL of the organic layer and evaporate it to dryness, reconstitute the residue in 500 μL 100 mM perchloric acid, dilute 1:200, inject an aliquot.

HPLC VARIABLES

Column: 250 × 4.6 5 μm Biophase 6032 octyl (Bioanalytical Systems)

Mobile phase: n-Propanol:100 mM pH 5.0 sodium acetate 12.5:87.5

Flow rate: 1

Detector: E, Bioanalytical Systems LC-154, TL-5A glassy carbon electrode -0.85 V, Ag/AgCl reference electrode

CHROMATOGRAM

Retention time: 15.5 (glutamic acid), 16 (serine), 17 (threonine), 18 (glycine), 19.5 (alanine)

KEY WORDS

derivatization

REFERENCE

Jacobs,W.A.; Kissinger,P.T. Nitroaromatic reagents for determination of amines and amino acids by liquid chromatography/electrochemistry, *J.Liq.Chromatogr.*, **1982**, *5*, 881–895.

SAMPLE

Matrix: solutions

Sample preparation: Mix 400 μL aqueous sample solution with 100 μL buffer and 500 μL 15 mM fluorenylmethyl chloroformate in acetone, let stand for 40 s, wash three times with 2 mL portions of pentane, inject an aliquot of the aqueous layer. (Prepare buffer by adjusting the pH of 1 M boric acid to 6.2 with NaOH.)

HPLC VARIABLES

Column: 125 × 4.6 3 μm ODS Hypersil

Mobile phase: Gradient. MeCN:MeOH:buffer 10:40:50 for 3 min, to 50:0:50 over 9 min.

Flow rate: 1.3 for 12 min, to 2 over 0.5 min

Injection volume: 10

Detector: F ex 250 (filter) em 320 (filter)

CHROMATOGRAM

Retention time: 4.5 (Asn), 4.8 (Gln), 5 (Asp), 5.5 (Ser), 6 (Glu), 7.5 (Gly), 8 (Thr), 8.7 (Arg), 10 (Ala), 10.5 (Tyr), 12.5 (Pro), 13 (Met), 14 (Val), 14.5 (Phe), 14.8 (Ile), 15 (Leu), 17.5 (His), 18 (Orn), 19.5 (Lys)

Limit of quantitation: 100 nM

KEY WORDS

derivatization

REFERENCE

Einarsson,S.; Josefsson,B.; Lagerkvist,S. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography, *J.Chromatogr.*, **1983**, *282*, 609–618.

SAMPLE

Matrix: solutions

Sample preparation: Mix amino acid solution with 2 mL freshly-prepared buffer, add 5 mL 10 ppm p-N,N-dimethylaminophenylisothiocyanate in acetone, purge tube with nitrogen, heat at 50° for 20 min, add 5 mL acid solution, purge with nitrogen, heat at 50° for 20 min, let stand for 20 min, evaporate to dryness under reduced pressure, wash the residue several times with isooctane, reconstitute with 100 mM pH 2 phosphate buffer, inject a 10 μL aliquot. (Prepare the buffer by mixing 50 mL water, 50 mL acetone, 5 mL 200 mM acetic acid, 1 mL triethylamine, and enough pyridine to adjust the pH to 9.5. Prepare the acid solution by mixing equal volumes of water and glacial acetic acid saturated with HCl. Synthesis of p-N,N-dimethylaminophenylisothiocyanate is as follows. Add 150 mL water containing 33 g sodium carbonate and 24 g thiophosgene to 60 mmoles N,N-dimethyl-1,4-phenylenediamine (p-N,N-dimethylaminoaniline) in 100 mL EtOH:water 50:50 with vigorous stirring, stir at room temperature for 2 h, extract with dichloromethane. Dry the organic layer and evaporate it to dryness under reduced pressure to obtain p-N,N-dimethylaminophenylisothiocyanate as an oil.)

HPLC VARIABLES

Column: 250 × 4.6 10 μm RP-8 (Hewlett-Packard)

Mobile phase: MeCN:18.13 g/L KH_2PO_4 25:75, adjusted to pH 2 with sulfuric acid or KOH

Flow rate: 2.5

Injection volume: 10

Detector: E, Bioanalytical Systems LC-4, TL-5 glassy carbon flow-cell 0.85 V, Ag/AgCl reference electrode

CHROMATOGRAM

Retention time: 4 (Cys), 5 (Asp), 6 (CysCys), 7 (Asn), 9.5 (Ser), 10 (Glu), 10.5 (Gln), 15 (Thr), 18 (Gly), 19 (His), 20 (Ala), 22 (Arg), 25 (Met), 29 (Pro), 34 (Val), 42 (Trp), 49 (Phe), 53 (Tyr), 61 (Lys), 70 (Ile), 74 (Leu)

Limit of detection: 0.2-0.6 ng

KEY WORDS

derivatization

REFERENCE

Mahachi,T.J.; Carlson,R.M.; Poe,D.P. p-N,N-Dimethylaminophenylisothiocyanate as an electrochemical label for high-performance liquid chromatographic determination of amino acids, *J.Chromatogr.*, **1984**, *298*, 279–288.

SAMPLE

Matrix: solutions

Sample preparation: Add 50 μL of a 50 mM solution in water to 100 μL 1% Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's Reagent) in acetone, mix, add 20 μL 1 M sodium bicarbonate, mix at 30-40° for 1 h, cool to room temperature, add 10 μL 2 M HCl, mix, dry in a vacuum desiccator over NaOH, reconstitute with 500 μL DMSO, inject a 5-10 μL aliquot.

HPLC VARIABLES

Column: 100 × 8 C18 (Waters)

Mobile phase: Gradient. MeCN:50 mM pH 3.0 triethylammonium phosphate from 10:90 to 50:50 over 1 h.

Flow rate: 2

Injection volume: 5-10

Detector: UV 340

CHROMATOGRAM

Retention time: 17.68 (L-Asp), 19.40 (L-Glu), 20.28 (D-Asp), 21.40 (L-Ala), 22.71 (D-Glu), 26.72 (D-Ala), 28.21 (L-Met), 34.66 (D-Met), 35.82 (L-Phe), 41.22 (D-Phe)

KEY WORDS
derivatization; chiral

REFERENCE
Marfey,P. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene, *Carlsberg Res.Commun.*, **1984**, *49*, 591–596.

SAMPLE
Matrix: solutions
Sample preparation: Evaporate a 500 μL aliquot of an aqueous solution containing 25 μmoles amino acids to dryness under a stream of air at 37°, reconstitute with 250 μL 1 M sodium bicarbonate solution, add 500 μL 1% 2,4-dinitrofluorobenzene in acetone, mix, heat at 50° for 1 h, cool to room temperature, add 200 μL 2 M HCl, dry in a vacuum desiccator over NaOH, reconstitute in 1 mL MeOH, pass dry HCl gas through the solution for 5 min, cap the vial, let stand for 3 h, evaporate to dryness, dry in a vacuum desiccator over NaOH, reconstitute in 400 μL DMSO. Remove a 50 μL aliquot and add it to 200 μL 50 mM pH 7.5 HEPES buffer, add 50 μL 10.6 mg/mL carboxypeptidase-Y (Carlsberg Biotechnology) in water, mix, let stand at room temperature for 3 h, add 300 μL DMSO, inject a 25 μL aliquot. (All amino acids are converted to their dinitrophenyl methyl ester derivatives. The enzyme hydrolyses only L-amino acid methyl esters (at the α-position) and so D-amino acids (methyl ester at the α-position) can be distinguished from L-amino acids (carboxylic acid at the α-position). Methyl esters of carboxylic acid groups other than α are not affected. No racemization occurs during derivatization and so the procedure can be used to assess racemization reactions.)

HPLC VARIABLES
Column: 100 × 8 radial compression C18 (Waters)
Mobile phase: Gradient. MeCN:50 mM pH 3.0 triethylammonium phosphate buffer from 25:75 to 50:50 over 40 min.
Flow rate: 2
Injection volume: 25
Detector: UV 350

CHROMATOGRAM
Retention time: 8.10 (β-methoxy-L-Asp), 8.76 (L-Ala), 9.93 (gamma-methoxy-L-Glu), 15.93 (L-Met), 20.91 (dimethoxy-D-Asp), 22.75 (methoxy-D-Ala), 23.78 (L-Phe), 24.68 (dimethoxy-D-Glu), 32.58 (methoxy-D-Met), 39.59 (methoxy-D-Phe)

KEY WORDS
derivatization; chiral

REFERENCE
Marfey,P.; Ottesen,M. Determination of D-amino acids. I. Hydrolysis of DNP-L-amino acid methyl esters with carboxypeptidase-Y, *Carlsberg Res.Commun.*, **1984**, *49*, 585–590.

SAMPLE
Matrix: solutions
Sample preparation: 20 μL Amino acid solution in 100 mM HCl + 30 μL 300 mM pH 9.5 borate buffer + 50 μL 5 mM reagent in MeCN, let stand for 5 min, inject a 5 μL aliquot. (Prepare reagent as follows. Add 40 mmoles p-bromoaniline in 40 mL MeCN dropwise with stirring to 50 mmoles N,N'-disuccinimidyl carbonate in 60 mL MeCN over 3-4 h, stir for 1 h, remove the MeCN by evaporation. Dissolve the residue in 100 mL ethyl acetate, wash with 1 M HCl, wash with 4% sodium bicarbonate, wash with water, dry over anhydrous sodium carbonate, evaporate to obtain succinimido p-bromophenylcarbamate, recrystallize form benzene/acetone (Caution! Benzene is a carcinogen!)

HPLC VARIABLES
Column: 150 × 4.6 Develosil ODS-5 (Nomura Chemical)

Mobile phase: MeOH:0.1% phosphoric acid 45:55
Flow rate: 1
Injection volume: 5
Detector: UV 250

CHROMATOGRAM
Retention time: 7 (His), 8 (Arg), 16 (Ser), 21.5 (Pro), 26 (Thr), 28 (Ala)
Limit of detection: 0.15-0.3 ng

OTHER SUBSTANCES
Also analyzed: tert-butylamine, diethylamine, diisobutylamine, diisopropylamine, isobutylamine (mobile phase MeCN:water 55:45)

KEY WORDS
derivatization

REFERENCE
Nimura,N.; Iwaki,K.; Kinoshita,T.; Takeda,K.; Ogura,H. Activated carbamate reagent as derivatizing agent for amino compounds in high-performance liquid chromatography, *Anal.Chem.*, **1986**, *58*, 2372–2375.

SAMPLE
Matrix: solutions
Sample preparation: Mix a 50 μL 5 mM amino acid solution with 50 μL 400 mM pH 9.0 sodium borate buffer, add 100 μL 16 mg/mL (+)-naphthylethyl isocyanate in dry acetone while vortexing, vortex for 30 s, let stand at room temperature for 5 min, centrifuge at 4000 g for 2 min. Remove a 150 μL aliquot of the supernatant and wash it three times with 1 mL portions of wash solution, inject an aliquot. (Prepare wash solution by mixing equal volumes of cyclohexane saturated with 400 mM pH 9.0 borate buffer and ether. Wash ether with ferrous sulfate to remove peroxides if methionine is present.)

HPLC VARIABLES
Column: 250 × 4.6 5 μm octadecyl Si100 Polyol (Serva)
Mobile phase: MeCN:50 mM pH 6.2 ammonium acetate 15:85 (A) or 20:80 (B) or 25:75 (C) or 27.5:72.5 (D) or 35:65 (E) or 40:60 (F) or MeCN:100 mM acetic acid 20:80 (G)
Flow rate: 1
Detector: F ex 214 em 320 (cut-off filter)

CHROMATOGRAM
Retention time: 22.6 (D-Asp (G)), 24.4 (L-Asp (G)), 26.6 (D-Glu (G)), 28.2 (L-Glu (G)), 20.8 (D-Asn (A)), 23.2 (L-Asn (A)), 19.3 (D-Ser (A)), 20.8 (L-Ser (A)), 27.6 (D-His (A)), 31.5 (L-His (A)), 9.9 (D-Thr (B)), 11.9 (L-Thr (B)), 11.0 (D-Pro (B)), 12.2 (L-Pro (B)), 11.7 (D-Ala (B)), 13.2 (L-Ala (B)), 6.9 (D-Arg (C)), 7.7 (L-Arg (C)), 10.4 (D-Val (C)), 13.2 (L-Val (C)), 5.8 (D-Met (D)), 7.4 (L-Met (D)), 9.7 (D-Ile (D)), 12.1 (L-Ile (D)), 11.0 (D-Leu (D)), 13.5 (L-Leu (D)), 16.0 (D-Trp (D) (UV 215)), 18.3 (L-Trp (D) (UV 215)), 17.3 (D-Phe (D)), 20.6 (L-Phe (D)), 15.5 (D-Lys (E)), 17.8 (L-Lys (E)), 17.3 (D-Tyr (F)), 19.0 (L-Tyr (F))
Limit of detection: <1 pmole

KEY WORDS
derivatization; chiral

REFERENCE
Dunlop,D.S.; Neidle,A. The separation of D/L amino acid pairs by high-performance liquid chromatography after precolumn derivatization with optically active naphthylethyl isocyanate, *Anal.Biochem.*, **1987**, *165*, 38–44.

SAMPLE
Matrix: solutions

Sample preparation: Mix a 20 μL aliquot of a solution in 100 mM HCl with 20 μL 500 mM pH 9.5 borate buffer and 20 μL 1.5 mg/mL reagent, mix for 1.5 min, let stand for 3 min, inject a 6 μL aliquot. (Prepare the reagent, succinimido naphthylcarbamate, as follows. Add 40 mL 1 M 1-naphthylamine in MeCN dropwise to 60 mL MeCN containing 50 mmoles N,N'-disuccinimidyl carbonate with stirring at room temperature over 3-4 h, after addition is complete stir for 1 h at room temperature, evaporate to dryness, dissolve the residue in 100 mL ethyl acetate. Wash the solution with 1 M HCl, wash with 4% sodium bicarbonate, wash with water, dry over anhydrous sodium sulfate, evaporate to dryness, recrystallize from benzene/acetone to obtain succinimido naphthylcarbamate (Caution! Benzene is a carcinogen!) (Anal.Chem. 1986, 58, 2372).)

HPLC VARIABLES

Column: 10 × 6 Develosil ODS-5
Mobile phase: Gradient. MeCN:100 mM pH 6.30 sodium acetate from 14:86 to 16:84 over 5 min, to 25:75 over 10 min, to 39:61 over 6 min, to 70:30 (step gradient), maintain at 70:30 for 6 min, re-equilibrate at initial conditions for 13 min.
Column temperature: 30
Flow rate: 1.2
Injection volume: 6
Detector: F ex 290 em 370

CHROMATOGRAM

Retention time: 3.5 (Asp), 4 (Glu), 8 (Ser), 8.4 (His), 8.8 (Pro), 9.2 (Gly), 9.6 (Arg), 10 (Thr), 11 (Ala), 15 (Tyr), 16 (ammonia), 16.7 (Val), 17.5 (Met), 19.5 (Ile), 20 (Leu), 21.5 (Phe), 22 (Cys), 24.5 (Lys)
Limit of detection: 75-1200 fmole

KEY WORDS

derivatization

REFERENCE

Iwaki,K.; Nimura,N.; Hiraga,Y.; Kinoshita,T.; Takeda,K.; Ogura,H. Amino acid analysis by reversed-phase high-performance liquid chromatography. Automatic pre-column derivatization with activated carbamate reagent, *J.Chromatogr.*, **1987**, *407*, 273–279.

SAMPLE

Matrix: solutions
Sample preparation: Mix 10 μL of an amino acid solution in water with 30 μL buffer, add 20 μL 50 mg/mL sodium 1-thio-β-D-glucose, add 20 μL 40 mg/mL o-phthalaldehyde in MeOH, stir thoroughly for 1 min, add 120 μL 50 mM pH 6.05 sodium acetate, inject a 10 μL aliquot. (Prepare buffer by dissolving 500 mg boric acid in 19 mL water and adjusting the pH to 10.40 with KOH solution (45 g KOH in 100 mL water).)

HPLC VARIABLES

Column: 250 × 4 5 μm LiChrosorb RP-8
Mobile phase: Gradient. A was 50 mM sodium acetate adjusted to pH 6.05 with acetic acid. B was MeOH:100 mM pH 7.60 sodium acetate 90:10. A:B 100:0 for 8 min, to 45:55 over 47 min, wash with 0:100 for 5 min.
Flow rate: 1.2
Injection volume: 10
Detector: F ex 360 em 420 (cut-off filter)

CHROMATOGRAM

Retention time: 6.4 (L-Asp), 5.2 (D-Asp), 14.4 (L-Glu), 15.6 (D-Glu), 18.2 (L-Ser), 20.1 (D-Ser), 25.0 (L-Thr), 28.1 (D-Thr), 29.0 (L-Arg), 30.3 (D-Arg), 31.6 (L-Ala), 34.0 (D-Ala), 37.0 (L-Tyr), 37.3 (D-Tyr), 48.0 (L-Val), 46.4 (D-Val), 46.1 (L-norvaline), 47.2 (D-norvaline), 47.9 (L-Trp), 48.7 (D-Trp), 51.5 (L-Phe), 50.0 (D-Phe), 53.9 (L-Ileu), 52.8 (D-Ileu), 54.0 (L-Leu), 54.9 (D-Leu), 54.3 (L-norleucine), 55.2 (D-norleucine)

Limit of detection: <1 pmole

KEY WORDS
derivatization; chiral

REFERENCE
Jegorov,A.; Triska,J.; Trnka,T.; Cerny,M. Separation of α-amino acid enantiomers by reversed-phase high-performance liquid chromatography after derivatization with *o*-phthaldialdehyde and a sodium salt of 1-thio-β-D-glucose, *J.Chromatogr.*, **1988**, *434*, 417–422.

SAMPLE
Matrix: solutions
Sample preparation: Evaporate 10 μL of a 2.5 mM solution in 100 mM HCl to dryness under reduced pressure, reconstitute with 100 μL MeCN:pyridine:triethylamine:water 50:25:10:15, evaporate to dryness, reconstitute with 100 μL MeCN:pyridine:triethylamine:water 50:25:10:15, add 5 μL phenylisothiocyanate, mix, let stand at room temperature for 5 min, evaporate to dryness, reconstitute with 250 μL MeCN:water 20:70, inject an aliquot.

HPLC VARIABLES
Guard column: ODS-5 (Altex)
Column: 250 × 4.6 5 μm Ultrasphere ODS
Mobile phase: Gradient. A was 115 mM pH 6.0 ammonium acetate. B was 230 mM pH 6.0 ammonium acetate in MeCN:MeOH:water 44:10:46. A:B from 100:0 to 85:15 over 15 min, to 50:50 over 15 min, to 100:0 over 4 min, maintain at 100:0 for 3 min, return to initial conditions over 13 min.
Flow rate: 1
Detector: UV 254

CHROMATOGRAM
Retention time: 9 (Glu), 11 (Asp), 12.5 (Ser), 16.3 (Hyp), 17.3 (Gly), 17.7 (Gln), 18.5 (Asn), 22 (His), 23 (Thr), 23.6 (Ala), 24.6 (Arg), 24.8 (Pro), 32.5 (Tyr), 33.8 (Val), 35 (Met), 35.8 (Cys), 37.4 (Ile), 37.6 (Leu), 38.6 (Phe), 39 (Trp), 39.4 (Lys)
Limit of detection: 50-500 pmole

KEY WORDS
derivatization; comparison with other derivatization procedures

REFERENCE
McClung,G.; Frankenberger,W.T.,Jr. Comparison of reverse-phase high-performance liquid chromatographic methods for precolumn-derivatized amino acids, *J.Liq.Chromatogr.*, **1988**, *11*, 613–646.

SAMPLE
Matrix: solutions
Sample preparation: 2 mL 1 mM Amino acids in 40 mM pH 9.5 lithium carbonate buffer + 1 mL 5.56 mM dansyl chloride in MeCN, shake gently for 2 min, let stand at room temperature for 35 min, add 100 μL 2% ethylamine, inject a 20 μL aliquot. (Purify MeCN by distillation from dansyl chloride.)

HPLC VARIABLES
Guard column: ODS-5 (Altex)
Column: 250 × 4.6 5 μm Ultrasphere ODS
Mobile phase: Gradient. MeCN:30 mM pH 7.6 sodium phosphate buffer 10:90 for 0.1 min, to 45:55 over 22.9 min, return to initial conditions over 7 min.
Flow rate: 2
Injection volume: 20
Detector: UV 250

CHROMATOGRAM

Retention time: 9.5 (Asp), 10.5 (Glu), 17 (Asn, Hyp), 18.5 (Ser), 14.8 (Thr), 15.2 (Gly), 15.5 (Ala), 16 (Pro), 17 (Val), 18 (Met), 18.5 (Leu, Ile), 19 (Phe), 19.3 (Trp), 19.5 (Cys), 24 (Lys), 26 (Thr)

Limit of detection: 50-500 pmole

KEY WORDS

derivatization; comparison with other derivatization procedures; protect from light

REFERENCE

McClung,G.; Frankenberger,W.T.,Jr. Comparison of reverse-phase high-performance liquid chromatographic methods for precolumn-derivatized amino acids, *J.Liq.Chromatogr.*, **1988**, *11*, 613–646.

SAMPLE

Matrix: solutions

Sample preparation: 35 μL Solution of amino acids in water + 35 μL 3.3 mg/mL o-phthalaldehyde in MeOH:water 50:50 + 35 μL 4 mg/mL N-acetyl-L-cysteine in MeOH:water 50:50 + 175 μL 400 mM pH 9.4 potassium borate buffer, mix well, let stand at room temperature for at least 2 min, neutralize with 140 μL 1 M pH 3.5 sodium phosphate buffer, inject an aliquot.

HPLC VARIABLES

Column: 250 × 4 5 μm Nucleosil-120-C18 or 125 × 4 3 μm Nucleosil-120-C18

Mobile phase: MeOH:buffer 40:60 (Buffer was 2.5 mM copper(II) acetate containing 5 mM L-proline adjusted to pH 6.0 with ammonium acetate.)

Column temperature: 40

Flow rate: 1

Injection volume: 20

Detector: F ex 338 (bandpass filter) em 415 (longpass filter)

CHROMATOGRAM

Retention time: k' 1.8 (L-valine), k' 2.4 (D-valine), k' 2.8 (L-leucine), k' 3.2 (D-leucine)

OTHER SUBSTANCES

Also analyzed: other amino acids, α-alkylamino acids, amino acid amides

KEY WORDS

derivatization; chiral

REFERENCE

Duchateau,A.; Crombach,M.; Kamphuis,J.; Boesten,W.H.J.; Schoemaker,H.E.; Meojer,E.M. Determination of the enantiomers of α-H-α-amino acids, α-alkyl-α-amino acids and the corresponding acid amides by high-performance liquid chromatography, *J.Chromatogr.*, **1989**, *471*, 263–270.

SAMPLE

Matrix: solutions

Sample preparation: Dissolve amino acids in 20 μL 50 mM pH 9.0 sodium bicarbonate, add 40 μL 4 mM dabsyl chloride in MeCN, heat at 70° for 10 min, dry under vacuum, dissolve the residue in EtOH:water 70:30 (J. Chromatogr. 1985, 349 77), inject a 5 μL aliquot.

HPLC VARIABLES

Guard column: 20 × 4.6 5 μm Supelcosil LC-18

Column: 150 × 4.6 3 μm Supelcosil LC-18

Mobile phase: Gradient. A was 25 mM pH 6.8 KH_2PO_4. B was MeCN:isopropanol 75:25. A:B 80:20 for 1 min, to 77:23 over 3 min, maintain at 77:23 for 5 min, to 73:27 over 1 min,

maintain at 73:27 for 4 min, to 65:35 over 5 min, to 40:60 over 6 min, to 70:30 over 1 min, maintain at 30:70 for 3 min, re-equilibrate at initial conditions for 6 min.
Flow rate: 2
Injection volume: 5
Detector: UV 436

CHROMATOGRAM

Retention time: 4 (aspartic acid), 4.5 (cysteamine), 5 (glutamic acid), 5.7 (carboxymethylcysteine), 6 (S-sulfocysteine), 8.5 (asparagine), 9.5 (glutamine), 10 (serine), 10.7 (threonine), 11.4 (glycine), 12 (alanine), 12.5 (arginine), 13.5 (taurine), 14 (proline), 14.5 (valine), 16.4 (methionine), 17.2 (isoleucine), 17.7 (leucine), 18.2 (tryptophan), 18.5 (norleucine), 19 (phenylalanine), 20 (ammonia), 20.5 (cystine), 22.4 (OH-Lys), 23 (lysine), 23.5 (histidine), 24.3 (tyrosine)

KEY WORDS

derivatization

REFERENCE

Stocchi,V.; Piccoli,G.; Magnani,M.; Palma,F.; Biagiarelli,B.; Cucchiarini,L. Reversed-phase high-performance liquid chromatography separation of dimethylaminoazobenzene sulfonyl- and dimethylaminoazobenzene thiohydantoin-amino acid derivatives for amino acid analysis and microsequencing studies at the picomole level, *Anal.Biochem.*, **1989**, *178*, 107–117.

SAMPLE

Matrix: solutions
Sample preparation: Add a 10-fold excess of 1,1-diphenylborinic acid to a solution of amino acids in 1 M acetic acid in isopropanol:water 50:50, heat at 65-70° for 15-20 min, dry under reduced pressure, reconstitute with mobile phase, inject an aliquot. (Prepare 1,1-diphenylborinic acid as follows. Stir diphenylborinic acid ethanolamine ester in 1 M HCl under nitrogen for 30 min, extract with dichloromethane. Wash the organic layer twice with water, wash with brine, dry over anhydrous sodium sulfate, evaporate under reduced pressure, take up the oil in warm hexane, cool, collect and wash the crystals, recrystallize from hexane to obtain 1,1-diphenylborinic acid (mp 128-130°) (Biomed. Mass Spec. 1984, 11, 611).)

HPLC VARIABLES

Column: 250 × 4 Zorbax PTH
Mobile phase: MeCN:THF:6 mM pH 3.30 phosphoric acid 18:16:66
Detector: UV 254

CHROMATOGRAM

Retention time: 8.56 (phosphorylated serine), 10.78 (phosphorylated threonine), 11.33 (His), 13.77 (Asn), 14.22 (Gln), 14.63 (Gla), 15.53 (Ser), 15.63 (phosphorylated tyrosine), 16.13 (Asp), 16.63 (Gly), 16.94 (Thr), 17.12 (Glu), 18.30 (N-methylglycine), 18.75 (Ala), 19.12 (Pro), 20.70 (N,N-dimethylglycine), 21.97 (biocytin), 25.96 (Cys), 27.54 (gamma-methylglutamic acid), 28.49 (Val), 29.40 (glutathione), 29.99 (Tyr), 30.76 (Lys), 32.43 (Arg), 33.16 (diphthine), 34.52 (Met), 37.64 (Ile), 40.18 (Leu), 45.3 (Phe), 48.43 (Trp), 75.97 (S-(p-nitrobenzyl)glutathione), 89.69 (2,6-diaminopimelic acid), 90.60 (oxidized glutathione), 102.51 (glutamyllysine)

OTHER SUBSTANCES

Noninterfering: amines, peptides

KEY WORDS

derivatization

REFERENCE

Strang,C.J.; Henson,E.; Okamoto,Y.; Paz,M.A.; Gallop,P.M. Separation and determination of α-amino acids by boroxazolidone formation, *Anal.Biochem.*, **1989**, *178*, 276–286.

SAMPLE

Matrix: solutions

Sample preparation: Mix 500 μL of a 10 μM solution of amino acids in 100 mM pH 9.3 borax buffer containing 1 mM disodium EDTA with 500 μL 20 mM DBD-F in MeCN, heat at 50° for 30 min, cool in ice, inject a 1-10 μL aliquot. (Synthesis of DBD-F is as follows. Dissolve 0.5 g magnesium sulfate heptahydrate and 6 g NaOH in 60 mL water, throughout the reaction keep the flask at about 20° with cold water cooling, add 15 mL 30% hydrogen peroxide, add 75 mL MeOH, add 12.1 g powdered benzoyl peroxide in one go, stir for 10 min, pour into 150 mL 20% sulfuric acid, extract three times with 50 mL portions of chloroform, determine peroxybenzoic acid concentration by iodometric titration (Tetrahedron 1967, 23, 3327). Slowly add 110 mL 1 M peroxybenzoic acid in chloroform to 7 g 2,6-difluoroaniline dissolved in 100 mL chloroform, stir at room temperature, when reaction is complete (iodometric titration) wash with 2% sodium thiosulfate, wash with 5% sodium carbonate, wash with water, dry over anhydrous sodium sulfate, evaporate to dryness under reduced pressure, recrystallize 2,6-difluoronitrosobenzene form EtOH (mp 108.5-109.5). Stir 8.5 g 2,6-difluoronitrosobenzene in 85 mL DMSO at room temperature and add a solution of 3.91 g sodium azide in 85 mL DMSO dropwise, let stand for about 1 h, add to a large volume of water, extract with ether, dry the extracts over anhydrous sodium sulfate, evaporate to dryness under reduced pressure and distil to give 4-fluoro-2,1,3-benzoxadiazole as a colorless oil (bp 83°/12 mm Hg) (J.Chem.Soc.(C) 1970, 1433). Add 11 mL chlorosulfonic acid dropwise to 3 g 4-fluoro-2,1,3-benzoxadiazole in 10 mL chloroform at 0-10° (use a calcium chloride drying tube), stir at room temperature for 1 h, reflux for 2 h, cool, slowly pour into ice water, remove the organic layer, extract the aqueous layer with chloroform, combine the organic layer, wash, dry over anhydrous magnesium sulfate, evaporate under reduced pressure, take up the residue in 5 mL benzene (Caution! Benzene is a carcinogen!), chromatograph on a 150 × 30 column of silica gel (100-200 mesh Kanto Chemical) with n-hexane:benzene 50:50, evaporate the appropriate fractions to give 4-(chlorosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (CBD-F) as pale yellow needles (mp 64-66°) (Anal. Chem. 1984. 56, 2461). Stir 0.76 g CBD-F in 70 mL MeCN at 0-10° and add 1 g dimethylamine hydrochloride in 10 mL 100 mM pH 10 borax dropwise, adjust pH to 5 with 1 M HCl, concentrate to about 10 mL under reduced pressure, extract three times with 200 mL portions of diethyl ether, wash with water, dry over anhydrous magnesium sulfate, evaporate under reduced pressure, chromatograph on a 500 × 20 column of silica gel with chloroform, isolate the appropriate fraction and re-chromatograph on the same column with ethyl acetate:benzene 1:2 to give 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) as white needles (mp 124-125°) (yield = 1% !). On a Merck no. 5714 60F_{254} tlc plate eluted with chloroform DBD-F has Rf 0.32 and lies between two other reaction products (Analyst 1989, 114, 413). It is also reported that DBD-F can be purchased from Tokyo Kasei (TCI America, Portland OR).)

HPLC VARIABLES

Guard column: 20 × 3.9 37-50 μm Bondapak C18/Corasil

Column: 300 × 3.9 10 μm μBondapak C18

Mobile phase: Gradient. A was 0.1% trifluoroacetic acid in water. B was 0.1% trifluoroacetic acid in MeCN. A:B from 90:10 to 30:70 over 1 h.

Column temperature: 40

Flow rate: 1

Injection volume: 1-10

Detector: F ex 450 em 590

CHROMATOGRAM

Retention time: 10 (hydroxyproline), 12 (glycine), 15 (alanine), 19 (proline), 23 (valine), 27 (isoleucine), 28 (phenylalanine), 30 (lysine), 35 (tyrosine)

Limit of detection: 0.11-0.79 pmole

KEY WORDS
derivatization

REFERENCE
Toyo'oka,T.; Suzuki,T.; Saito,Y.; Uzu,S.; Imai,K. Evaluation of benzofurazan derivatives as fluorigenic reagents for thiols and amines using high-performance liquid chromatography, *Analyst*, **1989**, *114*, 1233–1240.

SAMPLE
Matrix: solutions
Sample preparation: Dry an aqueous solution of amino acids into a tube, add 10 μL reagent, vortex vigorously, let stand for 10 min, add 40 μL water, wash with 50 μL toluene, inject a 1-10 μL aliquot of he aqueous layer. (Prepare reagent by mixing 490 μL 50' mM 4-nitrophenyl isothiocyanate in MeCN, 50 μL 10% triethylamine in MeCN, and 50 μL water. Purify commercial 4-nitrophenyl isothiocyanate by sublimation at 96° and 13 mm Hg to give a pale yellow powder (mp 106.5°).)

HPLC VARIABLES
Column: 300 × 3.9 Pico-Tag free amino acid analysis column (Waters)
Mobile phase: Gradient. A was MeCN:140 mM sodium acetate:triethylamine 6:94:0.05, pH adjusted to 6.4 with acetic acid. B was MeCN:water 60:40. A:B from 85:15 to 40:60 over 20 min, to 0:100 (step gradient), maintain at 0:100 for 5 min, re-equilibrate at initial conditions for 12 min.
Column temperature: 46
Flow rate: 1
Injection volume: 1-10
Detector: UV 254

CHROMATOGRAM
Retention time: 3.3 (Asp), 3.5 (Glu), 7.2 (Ser), 8 (Gly), 8.3 (His), 9 (Arg), 9.8 (Thr), 10 (Ala), 10.7 (Pro), 12.2 (Tyr), 13.3 (Val), 14 (Met), 15.5 (Ile), 15.7 (Leu), 16 (cystine), 16.8 (Phe), 21.7 (Lys)
Limit of detection: 0.5-1 pmole

KEY WORDS
derivatization

REFERENCE
Cohen,S.A. Analysis of amino acids by liquid chromatography after precolumn derivatization with 4-nitrophenylisothiocyanate, *J.Chromatogr.*, **1990**, *512*, 283–290.

SAMPLE
Matrix: solutions
Sample preparation: Mix sample:50 (?) mM NaCN in 50 mM pH 9.3 borate buffer:25 (?) mM naphthalene-2,3-dicarboxaldehyde in MeOH 3:1:1, let stand for 15 min, inject a 50 μL aliquot.

HPLC VARIABLES
Column: 200 × 3 5 μm Chromspher ODS-2 C18 (Chrompack)
Mobile phase: Gradient. A was 50 mM pH 7.0 sodium phosphate buffer. B was MeOH: THF:water 50:20:30. A:B from 25:75 to 0:100 over 75 min.
Flow rate: 0.5
Injection volume: 50
Detector: F ex 420